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REFLECTIONS ON THE RELATIONSHIP OF MEDICAL RESEARCH TO THE PHYSICAL AND BIOLOGICAL SCIENCES

WE have been living through one of the great historical epochs, and, as so often has happened in the past, the actors and the audience have been largely unaware of the intense dramatic quality of the events. It has come about because the physical sciences eventually have become developed to such a degree that they are able to provide the means for the investigation and the basis for the understanding of the events going on in living organisms. For us as living organisms there could hardly be a more significant advance in knowledge.

Of course this concept has had a long and distinguished history in the mechanistic philosophy of life, and one can think, for example, of Harvey, Descartes, Borelli as having made great contributions in the seventeenth century; but their contributions could utilize only the crude physical concepts of their time, levers, pumps, valves and pipes, in explaining the large-scale mechanical events in a living organism. Not until the middle of the nineteenth century was it beginning to be realized that living organisms were composed of multitudes of living units, the cells; and it was not until the present century that this generalization was effectively utilized in investigations into the functioning of living organisms.

One example of very recent development would be the metabolic processes which are occurring in living cells, and which are responsible for the provision of their energy requirements and for all the chemical transformations that they bring about. This modern biochemical development is centred on the study of intracellular enzymes, and is hardly older than the 1920's. The fundamental syntheses of enzymatic activities in such cycles as the citric acid and the urea cycles is still more recent; and at the present time these chemical activities are being linked with the micro-structures in the cell, in particular with the microsomes that have been made visible by electron microscopy. It is impossible to overemphasize the significance of this new biochemistry for the study of metabolic disease.

Investigations on muscle contraction provide an excellent example of the refinement that is now possible in biology. Electron microscopy reveals the exquisite geometrical arrangement of the rod-like assemblages of the protein molecules, myosin and actin. These rods are cross-linked by chemical bonds, and this cross-linkage is modified by chemical events so as to produce the shortening that appears as a muscular contraction. Though much of this mechanism is still obscure, there has been an amazing conceptual advance—muscular contraction is being explained by changes in molecular forces between precise geometrical arrangements of known proteins. One can predict that this new information will be of great value in the investigation of muscle diseases such as the myopathies.

Many other fields of biology are also advancing rapidly, because of the success attending efforts to explain biological function by the properties of the participating molecules. A new science called molecular biology has arisen as recently as the 1950's. One of its most striking successes has been in the field of gene structure: the nature of the specific genetic information conveyed by the gene, the genetic code as it is called; the way in which this information is replicated, so that in each nuclear division the whole code is duplicated and hence preserved in its entirety; and the way in which the genetic information is employed as templates on which are synthesized the enzymes and other proteins. The exact chemical constitution of some simpler proteins is now known; Sanger, for example, was awarded

the Nobel Prize in Chemistry in 1958 for determining the structural formula of insulin. There are very recent examples of the clinical implications of this aspect of molecular biology in two inherited blood diseases, sickle cell anæmia and hæmoglobin C disease. It was shown last year that they were associated with a specific change in the hæmoglobin molecules. Two different amino acids are substituted for the glutamic acid normally occurring at one site in the hæmoglobin molecule, and this minute change presumably results from a single mutation in the gene concerned in this protein synthesis.

I cannot do better than to quote the conclusion reached by Perutz in his recent review in *Endeavour*:

> During the past six years, molecular biology has changed from a subject of speculation and uncertainty to an exact science. Powerful methods of X-ray analysis have led to the elucidation of the basic molecular patterns of the most important types of protein fibres and of deoxyribonucleic acid; they are now beginning to reveal the structure of globular proteins and small viruses.
>
> There is hope that genetic events in viruses and micro-organisms may soon be understood in molecular terms. A start has also been made in elucidating the genetic control and molecular mechanism of protein synthesis. Muscular contraction is another phenomenon which may soon be understood in molecular detail.

Many other examples of molecular biology could be given, and it could be claimed that almost all the interesting and fruitful investigations on the functioning of living organisms could properly be subsumed under this title. For example, the ionic mechanisms involved in the conduction of nerve impulses and the functioning of nerve cells have been elucidated by the application of the various micro-techniques to single cells; micro-electrode recording from the interiors of cells, radio-tracer techniques on single cells, and electron-microscopy.

The impressive successes of molecular biology indicate that the evolutionary process made full use of matter at the molecular level of organization. At this level matter provides the richness of properties and the potentiality for new developments that were essential for the amazing adaptability and variety of living organisms. Until this century we not only lacked the refined techniques that enabled living organisms to be investigated at the appropriate level, but we also lacked the requisite conceptual background, for until then the physical sciences had not advanced sufficiently far in their understanding of matter.

We may now ask: What bearing do all these new developments in the biological sciences have on medical research? If we define medical research as research bearing on the understanding and treatment of disease, until the end of last century almost all medical research would have been associated with the practice of medicine, and would have been carried out mainly in hospitals. Empirical methods were employed in the discovery of new methods of treatment, and the description of the manifestations of disease was almost entirely in terms of morbid anatomy. Now almost all the major discoveries relating to disease are made by scientists utilizing the new powerful experimental methods. This work still may be carried out in institutes attached to hospitals, but to an increasing extent the research institutes employ animals in all their investigations, and the application of the discoveries to the treatment of patients comes only at a late stage.

In searching for new methods of clinical treatment it is now rarely expedient to attempt trial-and-error methods on patients. Rather is it desirable to study experimentally the physiological, biochemical and cytological processes that are disordered in the disease and thus attempt to understand the nature of the pathological process. Eventually it may be hoped that from this study the essential nature of the disease will become apparent and treatment devised accordingly. At this stage applied pharmacology becomes important; but that, too, is largely dependent on the closely linked laboratory sciences of pharmacology and organic chemistry. In the last few decades impressive developments of pharmacology have been made possible by the organic chemists, and now there are whole arrays of

substances that act specifically on physiological functions. For example, there are the muscle relaxants, the anticholinesterases, the antihypertensives, the antihistaminics. The development of antibiotics further illustrates very well the interplay of highly developed technical procedures.

We come now to consider briefly the medical research institute that is designed on the basis of these new and revolutionary developments. A distinction should be drawn between medical research and medical sciences, though there is a great measure of overlap between them. Medical research is particularly orientated to the study of disease, while medical sciences are much broader in their scope, including the whole fields of such subjects as biochemistry, microbiology, physiology, pharmacology, genetics and cytology. In the medical sciences research is not related particularly to disease, though there is every justification for anticipating that it will have important bearing on the understanding and treatment of disease. In parenthesis, it should be noted that the John Curtin School of Medical Research in the Australian National University should more appropriately be known as a School of Medical Sciences.

It is not necessary that a medical research institute be attached to a hospital, but, in contrast with an institute of medical sciences, it is essential that good liaison with hospitals should exist, so that there is effective two-way traffic. Most of the research staff need not be medically qualified, but for effective liaison many should be, or at least be well informed on the clinical problems related to their field of interest. It is of paramount importance that there should be first-class chemists, physicists and physical chemists in the institute, for they command the knowledge and the technical procedures that are revealing so much about the functioning of living cells, both normally and in diseased states. The medical graduate with no additional scientific training is often now at a disadvantage, because he has so much to learn in the basic sciences of physics, chemistry and mathematics, as well as of such derivative sciences as physiology, biochemistry, microbiology, genetics and cytology. It is in this context that special mention should be made of the research courses now available for medical students in one or other of the preclinical sciences. By interrupting his professional course for one or two years, the medical student has an opportunity to undergo a preliminary training as a scientist and to discover if he has a special aptitude and liking for research. He then completes the medical course and subsequently may return to research. Already such medical science courses have attracted many able medical students into the basic medical sciences, or into medical research proper.

It will be sufficiently evident that the collaboration of several research workers with diverse experience is the only way in which an adequate know-how can be brought to bear on some new basic problem. The building of such research teams is one of the important tasks of the head of a research department. It will be sufficiently evident that great technical resources are also essential for effective research—not only equipment that can be bought ready-made, but also workshops for the development and fabrication of new equipment.

All these essential requirements of personnel and equipment show why research has now become so expensive. Yet such has been the achievement of medical research that there is little public criticism of the rapidly rising scale of costs. No doubt it is generally realized that even now the cost of medical research is an extremely small fraction of the cost of disease. Of course, not all medical research pays dividends, but the successes of medical research pay handsomely for the aggregate cost.

What of the future? It is hard to envisage a limit to the expansion of medical science, in respect both of those employed and of the cost; and the prestige of medical research was never higher. The future seems assured because of the fine achievements of the great medical research workers of this century.

J. C. ECCLES.

SOME ACQUIRED HÆMOLYTIC ANÆMIAS OF CHILDHOOD[1]

JOHN HARLEY[2] AND LORIMER DODS[3]

From the Royal Alexandra Hospital for Children and the Institute of Child Health, Sydney

SUMMARY

A review has been made of the case records of eight children suffering from acquired hæmolytic anæmia, who were admitted to the Royal Alexandra Hospital for Children, Sydney, during the period 1954 to 1957.

Four of the eight children were of Mediterranean racial extraction. This incidence is significantly higher than the mean incidence of children of Mediterranean racial extraction in the Australian community.

Recovery from the illness was complete in each of the eight cases; in seven, the duration of the hæmolytic episode was less than four weeks. It is suggested that acquired hæmolytic anæmia in childhood is usually an acute disease of short duration.

In one case, excessive hæmolysis persisted for more than two years; for 16 weeks of this period, reticulocytopenia was present. The pathogenesis and prognosis of reticulocytopenia complicating auto-immune hæmolytic anæmia are discussed.

In one case the hæmolytic anæmia followed closely upon ingestion of fresh broad beans. Inquiry has established the fact that the common broad bean grown in Australia is *Vicia fava*. The glutathione content of the erythrocytes of this child, estimated more than one year after the hæmolytic episode, was found to be significantly reduced.

The significance of glutathione deficiency of the erythrocytes, in cases of acquired hæmolytic anæmia following ingestion of *Vicia fava*, naphthalene, sulphonamides and para-amino salicylic acid, is briefly discussed.

DURING the early years of the present century, Chauffard (1908) and other French hæmatologists suggested that there was an acquired form of hæmolytic anæmia which could be distinguished from the congenital form, and which was probably dependent on the presence of circulating hæmolysins: "*. . . il existe des faits dans lesquels l'état anémique semble subordonné à la présence d'hémolysins dans le sérum . . . les ictères hæmolysiniques.*" After the first World War this thesis and the names of its supporters seemed to be forgotten, and Lederer's description of three cases of acute hæmolytic anæmia which appeared in 1925 prompted the popular title "Lederer's anæmia", which was widely accepted for a decade or more as a diagnostic label for any acute acquired hæmolytic anæmia of childhood.

During the past 20 years, hæmatologists and pædiatricians have become increasingly aware of the wide range of ætiological factors which may be responsible for the development of an acquired hæmolytic anæmia during childhood, and the following records of eight children suffering from this type of disorder are presented with the object of illustrating certain aspects of this problem in pædiatric practice.

For the purposes of the present review, it has been assumed that transient anæmia, reticulocytosis and spherocytosis associated with either hyperbilirubinæmia or a positive direct reaction to the Coombs test justify a diagnosis of acquired hæmolytic anæmia.

The eight children whose records form the substance of this report were admitted to the wards of the Royal Alexandra Hospital for Children during the period 1954 to 1957, each of them suffering from a severe anæmia of rapid onset characterized by obvious spherocytosis and accompanied by a significant degree of reticulocytosis; hyperbilirubinæmia was evident in seven and hæmoglobinuria in three of these children. The direct Coombs test gave a positive result in four of the eight cases. In each case the anæmia, the reticulocytosis, the spherocytosis and the other hæmatological manifestations proved to be transient.

REPORTS OF CASES

CASE I.—M.V., a male child, aged three years, was admitted to hospital on November 22, 1956, with a history of having passed dark urine, in smaller amounts that usual, for three days, and of drowsiness, vomiting

[1] Received on June 18, 1958.

[2] Honorary Relieving Assistant Physician, Royal Alexandra Hospital for Children, Sydney.

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and jaundice for one day. There had been two previous episodes of jaundice, 18 months and six months before the present admission. The parents did not seek medical advice on these occasions, as the jaundice persisted for less than 24 hours and was unaccompanied by pallor or dark urine. The child was of Greek, Syrian and Egyptian racial extraction. He occasionally ate broad beans, but the parents could not recall ingestion of beans immediately before the onset of the illness. Naphthalene was the only other known noxious agent to which this child might have been exposed, and the parents considered that he could have gained access to the mothballs which were kept in the home.

On admission to hospital, he was very pale and jaundiced, and appeared to be seriously ill. The liver edge was palpable one centimetre below the right costal margin, and was tender. The spleen was palpable one centimetre below the left costal margin. The urine was scanty in amount and dark red in colour. The results of laboratory investigations have been recorded in diagrammatic form in Figure I. Numerous

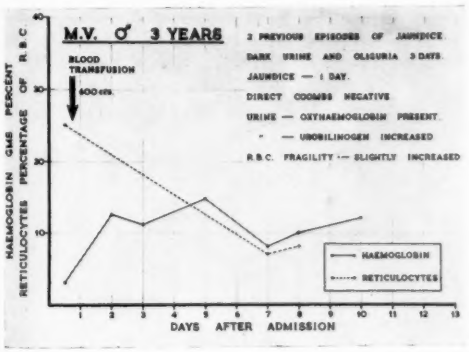

FIGURE I

Diagrammatic representation of the clinical and hæmatological features of Case I

spherocytes were detected in the peripheral blood, and the osmotic fragility of the erythrocytes was normal before incubation, and slightly increased after. The direct and indirect Coombs tests produced negative results.

After transfusion marked clinical and hæmatological improvement was noted, and the child was discharged, apparently well, 16 days after his admission to hospital. Eight months later, clinical examination and laboratory investigation failed to detect any abnormality.

CASE II.—S.C., a male infant, aged three years, of Italian racial extraction, was admitted to hospital on November 17, 1956. The clinical course was very similar to that in Case I. Although this child occasionally ate broad beans, his parents thought it unlikely that he had eaten any beans immediately before the onset of the illness, and they were unable to suggest any other ætiological factor. The results of laboratory investigations have been recorded in diagrammatic form in Figure II. Numerous spherocytes were detected in the peripheral blood, and the direct Coombs test produced a negative result.

The child improved rapidly after a blood transfusion, and was discharged, well, seven days after his admission. Eight months later, no clinical or hæmatological abnormality could be detected.

CASE III.—P.P., aged seven years, the son of Greek parents who had migrated from Egypt, was admitted to hospital on Wednesday, October 17, 1956. He was an intelligent boy who told his own story. One Sunday, while on a picnic, he consumed a large quantity of fresh broad beans. Several hours later, on the way home in the car, he vomited, then developed abdominal pain and alternating hot and cold shivers. On Monday he felt hot and stayed home from school; on Tuesday he was pale and yellow, and his urine was dark; on Wednesday he felt no better and was taken to hospital.

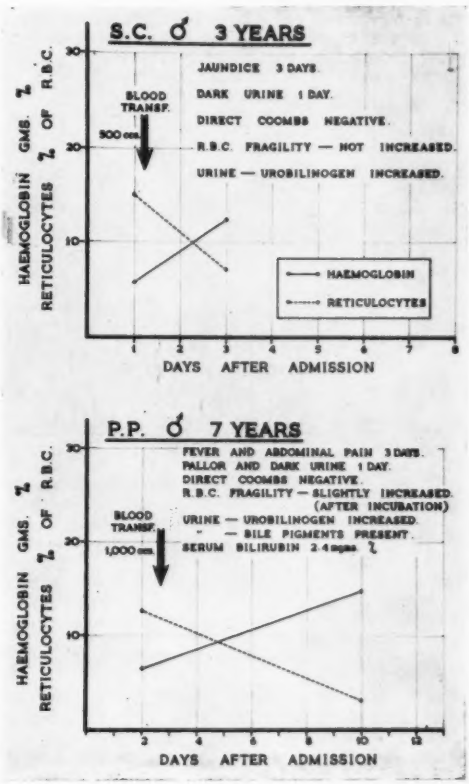

FIGURE II

Diagrammatic representation of the clinical and hæmatological features of Case II and Case III

His father recalls an incident in his own early childhood when, after eating broad beans, he vomited and became very ill; since then he has always refused to eat broad beans.

On examination, the child appeared to be seriously ill. Marked pallor and mild jaundice were noted. The liver edge, palpable one centimetre below the right costal margin, was not tender. The spleen was not palpable. The results of laboratory investigation were as follows. The hæmoglobin value was 6·9 grammes *per centum*, and the erythrocytes numbered 2,300,000 per cubic millimetre; reticulocytes numbered 290,000 per cubic millimetre, thus comprising 13%

of the erythrocytes; spherocytes in moderate numbers were present in the peripheral blood; the osmotic fragility of the erythrocytes was normal before incubation, and slightly increased after. The response to the direct Coombs test was negative. The serum bilirubin content was 2·4 milligrammes per 100 millilitres, small amounts of bile pigment were transiently present in the urine, and the urinary urobilinogen content was found to be slightly increased on one occasion (Figure II).

A transfusion of 1000 millilitres of blood was given on October 19 and was followed by marked clinical improvement. On October 26 the hæmoglobin value was 14·6 grammes *per centum*, reticulocytes comprised 3% of the erythrocytes, and spherocytes were no longer detected in the peripheral blood. The child was discharged from hospital, apparently well, on October 27.

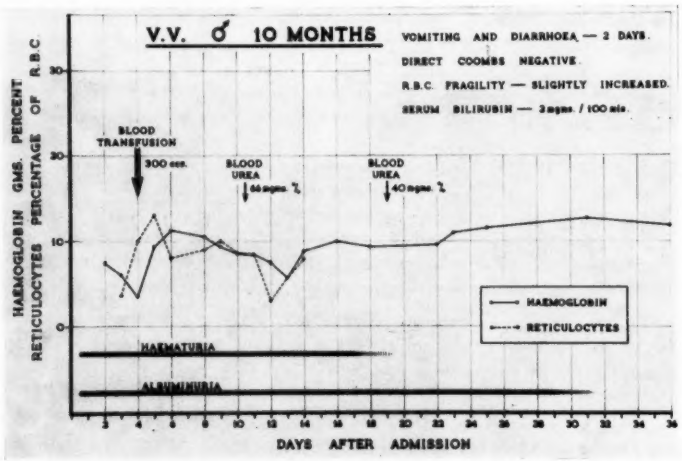

FIGURE III

Diagrammatic representation of the clinical and hæmatological features of Case IV

The child last attended the hospital on January 14, 1958. The hæmoglobin value was 12·1 grammes *per centum*, reticulocytes comprised 0·5% of the erythrocytes, and the morphology of the erythrocytes was normal. Examination of the blood by the alkali denaturation method revealed no fœtal hæmoglobin. No abnormality was detected on physical examination, and the parents stated that the boy had enjoyed excellent health since the illness. Both the child and his parents are adamant that he will never again eat broad beans.

The glutathione content of the erythrocytes of this child and of a number of normal children of the same age was determined by the nitroprusside method, as described by Thompson and Watson (1952).

The glutathione content of the erythrocytes collected from this patient on January 14, 1958, was 24·4 milligrammes per 100 millilitres of blood. Determination of the glutathione content of the erythrocytes of 11 normal children of the same age revealed levels of 48·8, 48·0, 47·2, 44·4, 39·6, 39·2, 38·8, 38·4, 38·0, 36·0 and 33·6 milligrammes per 100 millilitres of blood, with a mean of 41·09 milligrammes per 100 millilitres of blood ($P<0·01$).

In each of these three cases (I, II and III), the child was of Mediterranean racial extraction, the hæmolytic episode was of severe degree and brief duration, and the result of the direct Coombs test was negative. Broad beans had been eaten from time to time by each of the three children; subsequent inquiry has established the fact that the common broad bean grown in Australia is *Vicia fava*. The history in Case III indicates that the illness followed closely upon the ingestion of fresh broad beans and seems to justify a diagnosis of favism.

In Case I the ætiology remains unknown, but the fact that mothballs were known to have been within reach of this three-year-old infant raises the possibility of naphthalene poisoning.

CASE IV.—V.V., a male infant, aged 10 months, was admitted to hospital on April 2, 1956, with a history of vomiting and diarrhœa of five days' duration. The child's father was of Italian racial extraction, his mother British.

On examination, the child was noted to be pale and slightly dehydrated. Jaundice was first detected on the day after his admission to hospital. The clinical course and laboratory findings are shown in diagrammatic form in Figure III. Spherocytes were noted in the peripheral blood, and the direct and indirect

Coombs tests produced negative results. Microscopically evident hæmaturia persisted for nearly three weeks, and albuminuria for more than one month.

When he was examined again nearly one year after his discharge from hospital, the child appeared very well, and no abnormality could be detected on microscopic examination of the urine or on hæmatological investigation.

This ten-month-old infant was of Mediterranean racial extraction. The available evidence supports a diagnosis of acquired hæmolytic anæmia, and it is reasonable to assume that he was also suffering from acute glomerulonephritis. Microscopically evident hæmaturia persisted for nearly three weeks, and albuminuria for more than one month after his admission to hospital; the blood urea content was elevated to 66 milligrammes per 100 millilitres on one occasion, and both the direct and indirect Coombs tests produced negative results.

This association of acute hæmolytic anæmia with acute glomerulonephritis has been discussed by Hensley (1952) and Robertson (1957).

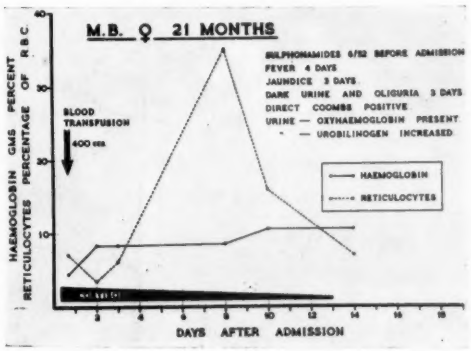

FIGURE IV

Diagrammatic representation of the clinical and hæmatological features of Case V

CASE V.—M.B., a female infant, aged 21 months, was admitted to hospital on January 26, 1954, with a history of fever for four days, of jaundice, oliguria and dark urine for three days, and of pallor for one day. The child had received short courses of sulphonamides on two occasions, three months and again six weeks before the onset of the illness, for the treatment of attacks of acute tonsillitis. The parents were of British racial extraction, and offered no relevant family history of disease.

On examination, the child was found to be lethargic, pale and jaundiced. The spleen was not palpable. The liver edge, palpable one centimetre below the right costal margin, was not tender. The results of laboratory investigation are represented in diagrammatic form in Figure IV. Spherocytes were detected in the peripheral blood, and the response to the direct Coombs test was positive.

Blood transfusion was administered soon after the child's admission to hospital, and cortisone was given for a period of 13 days. The child was discharged, apparently well, 17 days after admission to hospital. Clinical examination and laboratory investigation three years later failed to reveal any abnormality.

CASE VI.—A.C., a female infant, of British racial extraction, was admitted to hospital on August 15, 1953, at the age of 20 months. The clinical course was very similar to that of M.B. (Case V), with the exception that there were no known ætiological factors, and that therapy comprised the administration

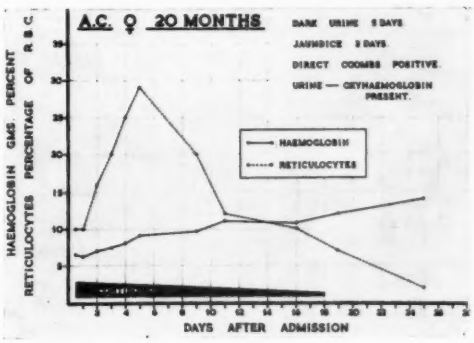

FIGURE V

Diagrammatic representation of the clinical and hæmatological features of Case VI

of cortisone alone, rather than cortisone and blood transfusion. The hæmatological findings in this case are represented diagrammatically in Figure V. Numerous spherocytes were detected in the peripheral blood, and the direct Coombs test produced a positive result.

The child was discharged from hospital apparently well 28 days after admission, and clinical and laboratory investigation four years later failed to reveal any abnormality.

These two female infants were of almost exactly the same age, and were both considered to be of British racial extraction. A number of further points of similarity between these two cases may be emphasized. The first is the severity of the illness, reflected by the clinical appearance of the infants on their admission to hospital, the marked degree of anæmia, and the presence of oxyhæmoglobin in the urine. The second is the positive response to the direct Coombs test. The third is the short duration of the episode of excessive hæmolysis, and the rapid and apparently complete recovery from the illness. The fourth is the absence of any known noxious agent which might have been responsible for the hæmolytic episode. A sulphonamide drug had been ingested by one of these patients (M.B., Case V) three months and again six weeks before the onset of the illness; but this drug was considered to be an

unlikely ætiological agent, in view of Dacie's observation that " hæmolysis usually takes place at an early stage of treatment, as a rule within 24 to 72 hours of taking the [sulphonamide] drugs ".

CASE VII.—V.K., a female child, aged eight years, of British racial extraction, was admitted to hospital on March 21, 1957. For 18 months she had suffered from attacks of *petit mal*, which had been well controlled by " Tridione " for the past nine months. For two weeks she had been feverish, listless and pale, and enlargement of the lymph nodes, liver and spleen had been noted by the local medical attendant.

On examination, the child was noted to be pale, but not jaundiced. The oral temperature was 102° F., and fine crepitations were audible at the base of the left lung. The cervical, axillary and inguinal lymph nodes were enlarged, discrete and mobile, but not tender. The liver, palpable three centimetres below the right costal margin, and the spleen, palpable two centimetres below the left costal margin, were tender and firm. A skiagram of the chest revealed opaque areas suggesting extensive consolidation of the lower lobe of the left lung. The results of laboratory investigations are shown in Figure VI. A moderate number of spherocytes were present in the peripheral blood. The leucocytes numbered 16,000 per cubic millimetre, with 91% neutrophils and no atypical mononuclear cells. The direct Coombs test produced a positive result. The possible significance of the coincidental pneumonia was unfortunately not recognized, and a specific search for cold agglutinins was not made. Heterophil agglutinins were present to a titre of 1 : 7 (Figure VI).

Tetracycline therapy was commenced soon after her admission to hospital, and a blood transfusion was given on March 25. On March 30 the hæmoglobin value was 12 grammes *per centum*, at which level it was maintained. Spherocytes were not detected in the peripheral blood after April 1. Despite the hæmatological improvement, the child was not well. Fever and clinical signs of pneumonia persisted in spite of the administration of broad-spectrum antibiotics, and further radiological opacities appeared at the base of the right lung. At no stage of the illness was jaundice apparent or the serum bilirubin content found to be increased. On March 21, the day of the child's admission to hospital, the zinc sulphate turbidity value of the serum was raised to 17 units (the normal range being one to six units), and the thymol turbidity test produced a negative result. Three weeks later the zinc sulphate turbidity value of the serum was 17 units, and there was a moderate increase in thymol flocculation.

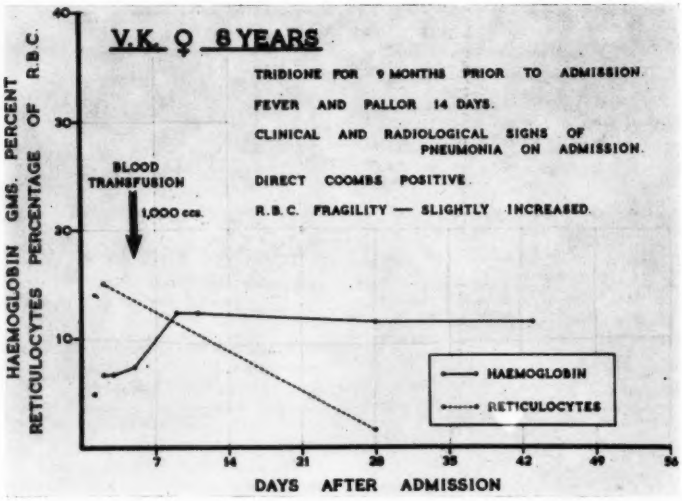

FIGURE VI
Diagrammatic representation of the clinical and hæmatological features of Case VII

Five weeks after her admission to hospital, the child's general condition began to improve, and the radiological opacities slowly disappeared. She was sent home, apparently well, on May 15, eight weeks after her admission to hospital. When she was last examined, on August 1, 1957, she said that she had been very well since the illness. The liver and lymph nodes were not enlarged, and the spleen was not palpable. The hæmoglobin value was 13·5 grammes *per centum*, reticulocytes comprised less than 1% of the erythrocytes, and the morphological characteristics of the erythrocytes were normal. The zinc sulphate turbidity value was 11 units, and paper electrophoresis of the serum indicated a slight increase in gamma globulin. The direct Coombs test still produced a positive result to antiglobulin titres of 1 : 4. Gamma globulin neutralization showed no decrease in titre, which indicated that the antibody was not a gamma globulin, and was therefore more likely to be of a cold antibody type.

The ætiology of the episode of excessive hæmolysis in this case remains obscure. It is known that "Tridione" may occasionally produce hepatitis or bone-marrow depression, but no report could be found of hæmolytic anæmia following ingestion of this drug. The absence of an abnormally high titre of heterophile agglutinins and of atypical mononuclear cells makes it unlikely that the hæmolytic anæmia was associated with infectious mononucleosis. The possible significance of the abnormal results of the liver function tests must be considered in the light of Dacie's statement, as follows:

> The so-called chemical tests of liver function cannot be relied on in the presence of hæmolytic anæmia, for changes in serum globulins are frequently encountered in hæmolytic anæmia quite irrespective of the presence or absence of liver disease.

It seems most probable that this child was suffering from primary atypical pneumonia. The paucity of physical signs in the chest, the prolonged course of the illness and the failure to respond to antibiotic therapy all support this suggestion. Interpretation of the leucocytosis is difficult in the presence of coincidental hæmolytic anæmia. The fact that the abnormal antibody was shown not to be a gamma globulin suggests that it is more likely to be of a cold antibody type, and gives further support to a diagnosis of primary atypical pneumonia and hæmolytic anæmia.

CASE VIII.—G.C., a female infant, of British racial extraction, was first admitted to hospital on June 3, 1952, at the age of 15 months, with the short history that she had been pale for four days, and that her urine and fæces had been dark for two days. No relevant personal or family history was obtained.

On examination of the child, pallor and jaundice were noted; the liver and spleen were not palpable. The results of laboratory investigations were as follows: The hæmoglobin value was 4·8 grammes *per centum*, and the erythrocytes numbered 1,340,000 per cubic millimetre; reticulocytes comprised 15% of the erythrocytes; erythroblasts numbered 6000 per cubic millimetre; numerous spherocytes were present in the peripheral blood. The serum bilirubin content was 4·5 milligrammes per 100 millilitres, and the urinary urobilinogen content was found to be increased on two occasions. The direct Coombs test produced a positive result (Figure VII).

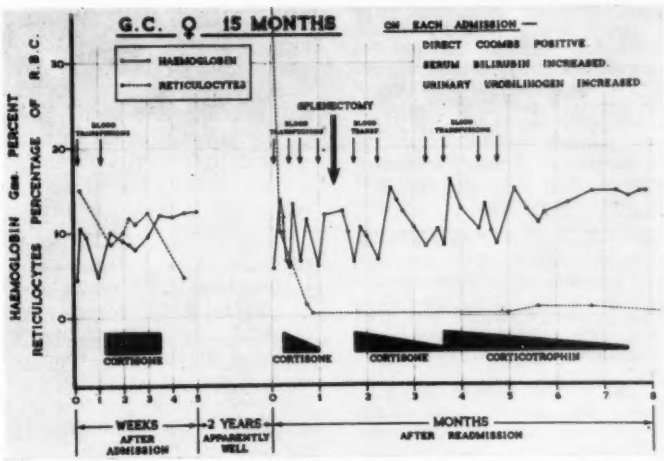

FIGURE VII

Diagrammatic representation of the clinical and hæmatological features of Case VIII

A transfusion of 300 millilitres of blood was given on June 3. The hæmoglobin value, which was 11 grammes *per centum* on June 4, had fallen to five grammes *per centum* on June 11. A second transfusion of 400 millilitres of blood was given, and the administration of cortisone was commenced, in daily dosage of 100 milligrammes. On June 26, the hæmoglonin value was 12 grammes *per centum*, the child appeared well, and steroid therapy was discontinued. On July 5, 32 days after her admission to hospital, the hæmoglobin value was 12 grammes *per centum*, reticulocytes comprised 5% of the erythrocytes, spherocytes were still present in the peripheral blood, and the result of the direct Coombs test was still positive. The child was discharged from hospital on July 8, 1952.

For the next 22 months she was pale and occasionally passed dark urine, but as she seemed well her parents were not sufficiently worried to seek medical advice. However, when she was readmitted to hospital on March 24, 1954, examination showed her to be a pale, jaundiced child, in whom the liver and spleen were palpable one centimetre below the costal margin, and

it was stated that she had been listless and very pale for two weeks and jaundiced for two days. On laboratory investigation, the hæmoglobin value was 5·4 grammes *per centum*, and the erythrocytes numbered 1,640,000 per cubic millimetre; reticulocytes comprised 40% of the erythrocytes, erythroblasts numbered 12,000 per cubic millimetre, and numerous spherocytes were detected in the peripheral blood. The result of the direct Coombs test was positive. The serum bilirubin content was 3·2 milligrammes per 100 millilitres, and the urinary urobilinogen content was found to be increased on numerous occasions (Figure VII).

After a transfusion of 1000 millilitres of blood, the hæmoglobin value rose to 14 grammes *per centum*, then fell to seven grammes *per centum* by March 31, the seventh day after admission to hospital. The administration of cortisone, in a daily oral dosage of 200 milligrammes, was then commenced, and continued until April 23, but excessive hæmolysis continued, and three transfusions of approximately 1000 millilitres of blood were necessary over this period.

Splenectomy was performed on April 29, just five weeks after the child's readmission to hospital. The spleen weighed 200 grammes, and microscopic examination showed the sinusoids and pulp to be packed with erythrocytes, and a moderate excess of hæmosiderin to be present.

On May 5, cortisone therapy was recommenced. The daily dosage was 200 milligrammes for two days, 100 milligrammes for 28 days, 75 milligrammes for seven days, 50 milligrammes for three days and 25 milligrammes for two days. Cortisone was then replaced by long-acting corticotrophin in a single daily dosage of 20 units for three months, followed by 10 units for one month.

Six transfusions, each of approximately 1000 millilitres of blood, were necessary in the four months after splenectomy. In August, after the sixth transfusion of the post-splenectomy period, the hæmoglobin value rose to 15 grammes *per centum*, fell to 11 grammes *per centum*, and then slowly rose to 15 grammes *per centum* without further transfusion. On November 9, corticotrophin therapy was discontinued, in view of the maintenance of a satisfactory hæmoglobin level without transfusion for a period of 10 weeks. The direct Coombs test, repeated on July 20, still produced a positive result. Spherocytes were present in the peripheral blood until the end of May, and then disappeared.

The reticulocytes are worthy of a special study. On the day of the child's readmission to hospital, March 24, 1954, reticulocytes comprised 40% of the erythrocytes. Five weeks later, on April 28, reticulocytes comprised 1·2% of the erythrocytes, and bone-marrow biopsy revealed marked erythroid hyperplasia. For the next 16 weeks the reticulocyte count, whenever determined, was found to be low; reticulocytes always comprised less than 0·5% of the erythrocytes; the reticulocyte count was always less than 30,000 and usually less than 6000 per cubic millimetre. On July 15, 14 weeks after her readmission, bone-marrow biopsy again revealed erythroblastic hyperplasia. By September 9, the reticulocyte count had risen to 50,000 per cubic millimetre, at which level it has been maintained. Since that time, adequate release of erythrocytes from the bone marrow has been reflected by the maintenance of the hæmoglobin value at normal levels without blood transfusion.

After the cessation of corticotrophin therapy on November 9 the child remained in good health, and she was discharged from hospital, apparently well, on November 23, 1954. When she attended again in May, 1955, the parents reported that she had been well since her illness. The hæmoglobin value was 13·8 grammes *per centum*, the reticulocytes numbered 50,000 per cubic millimetre and the results of the direct and indirect Coombs tests were negative. The child was last examined in March, 1957, when she had the appearance of a healthy child; her hæmoglobin value was 13 grammes *per centum*, and the morphological characteristics of the erythrocytes were normal.

This female infant suffered from a severe episode of auto-immune hæmolytic anæmia of five weeks' duration, followed by 22 months of comparatively good health, and then by a second, severe and prolonged episode of auto-immune hæmolytic anæmia. That excessive hæmolysis was taking place over the period of 22 months of comparatively good health is strongly suggested by the history of constant pallor during this period, the positive response to the direct Coombs test, reticulocytosis and spherocytosis at its commencement, and the overt evidence of a severe auto-immune hæmolytic anæmia at its completion.

Despite the continued administration of cortisone, four transfusions, each of approximately 1000 millilitres of blood, were necessary in the first four weeks of the second period in hospital, and splenectomy was performed at the end of the fifth week. Six transfusions, each of approximately 1000 millilitres of blood, were required in the four months after splenectomy. Thus four transfusions were necessary in one month before splenectomy and six transfusions in four months after, approximately the same volume of blood being administered on each occasion. This would seem to suggest that at about the time of splenectomy a considerable reduction in the rate of hæmolysis did occur, but it obviously does not establish the fact that the reduction in the rate of hæmolysis was the result of splenectomy.

Reticulocytopenia was evident for four months after splenectomy. Even when allowance is made for a diminished release of erythrocytes from the bone marrow, there is ample evidence that the rate of hæmolysis was still excessive over this period; not only did the hæmoglobin values frequently fall by more than two grammes *per centum* in less than one week, but the volume of blood required to maintain the hæmoglobin values within the normal range was much greater than would have been needed by a child of comparable weight suffering from complete marrow aplasia without increased hæmolysis.

Four months after splenectomy, the hæmoglobin values and reticulocyte counts slowly returned to levels within the normal range, and no further administration of blood was necessary.

For two years since the illness this child has enjoyed excellent health, and repeated hæmatological examinations have failed to reveal any abnormality.

A REVIEW OF THE CLINICAL AND HÆMATOLOGICAL FEATURES

Clinical Features

The ages of the eight children ranged from 10 months to eight years; four of these children were aged less than two years, and six of them less than four years.

Four of the eight children were of Mediterranean racial extraction. This incidence is significantly higher than the mean incidence of children of Mediterranean racial extraction in the Australian community, as, at the present time, only about 2·4% of the total Australian population are of Mediterranean birth (Commonwealth Department of Demography, personal communication, 1958).

The onset of the illness was usually abrupt. The early pattern of the disease, as described by the parents, was constant, being characterized by such clinical features as pallor, lassitude, fever, vomiting, abdominal pain, dark urine and jaundice. The duration of symptoms prior to the child's admission to hospital was short, being less than five days in seven cases.

On examination in hospital, each of the patients had the appearance of a seriously ill child. Pallor of severe degree was noted in every case. A slight to moderate degree of jaundice was present in seven cases. The spleen was palpable in only three cases.

The duration of the illness was short in seven of the eight cases, neither clinical nor laboratory evidence of excessive hæmolysis being evident 21 days after the patient's admission to hospital. Excessive hæmolysis apparently persisted for two years and eight months in Case VIII.

Laboratory Investigations

In every case the anæmia was severe, the hæmoglobin value falling to levels of three to seven grammes *per centum*, and the erythrocyte count to levels of 1,000,000 to 3,000,000 erythrocytes per cubic millimetre.

In every case a significant reticulocytosis accompanied the anæmia, the maximum reticulocyte counts ranging from 250,000 to 750,000 per cubic millimetre, and the reticulocytes comprising from 12% to 40% of the erythrocytes. Transient reticulocytopenia was noted in one patient. Erythroblastæmia was present in the five youngest of the eight children, the maximum absolute counts ranging from 1800 to 16,000 erythroblasts per cubic millimetre. Neutrophil leucocytosis and normal platelet counts were found in every case.

In every case a significant degree of spherocytosis was evident in the peripheral blood. In five of the six cases in which the resistance of the erythrocytes to hypotonic saline was determined, slightly increased fragility was detected. In the sixth case there was no increase in fragility either before or after incubation, and it is interesting to note that spherocytes were no longer detected in the peripheral blood of this patient when the osmotic fragility test was performed.

The anæmia, reticulocytosis and spherocytosis disappeared after each of the children had recovered from the illness. In every case laboratory investigation, performed more than six months after recovery from the illness, revealed a hæmoglobin value and reticulocyte count within the normal range, and erythrocytes of normal morphological characteristics.

The response to the direct Coombs test was positive in four and negative in four of the eight cases. Hyperbilirubinæmia was detected in seven of the eight cases, and oxyhæmoglobinuria was evident in three of these seven cases.

DISCUSSION

Duration of Illness

After a review of the literature, Larson (1957) concludes that "in childhood the acute type is dominating, while the chronic form seems to be rare". The present series supports this suggestion that acquired hæmolytic anæmia in childhood is usually a disease of short duration.

In seven of the eight cases in this series, the duration of the episode of excessive hæmolysis was less than four weeks; the interval between the onset of symptoms and the child's admission to hospital was short (less than five days in six cases); after the institution of therapy there was rapid clinical improvement, with return of the hæmoglobin values to normal levels; hyperbilirubinæmia and spherocytosis were transient; and within 21 days of the patient's admission to hospital there was no obvious evidence of increased hæmolysis.

On the other hand, in Case VIII the duration of excessive hæmolysis was almost certainly more than two years. For 22 months between two frank episodes of auto-immune hæmolytic anæmia, pallor was constant and the urine was occasionally noted to be dark. This is the only patient with acquired hæmolytic anæmia admitted to the Royal Alexandra Hospital for Children over the last five years in whom the writers are aware of the persistence of excessive hæmolysis for more than one month.

Prognosis

While no conclusion as to prognosis can be drawn from a series of eight cases, the fact that recovery from the disease was rapid and complete in seven children suggests that the prognosis of acquired hæmolytic anæmia in childhood is good. In Case VIII of the present series complete recovery finally occurred, despite the severe and prolonged hæmolysis, the poor initial response to cortisone and splenectomy and the complication of reticulocytopenia.

Reticulocytopenia

Reticulocytopenia in the presence of hæmolytic anæmia was noted in Case VIII. For a period of 16 weeks, during which there was ample evidence of excessive hæmolysis, the reticulocyte count was always less than 30,000, and usually less than 6000 per cubic millimetre.

Crosby and Rappaport (1956) studied 34 cases of idiopathic auto-immune hæmolytic anæmia, and discussed the significance of reticulocytopenia in this disease. Of 15 patients who were found to have relative reticulocytopenia at times of hæmolytic crisis, only three survived. The mortality in this group of patients with reticulocytopenia was significantly higher than in those who showed a reticulocyte response consistent with the severity of the anæmia, and the authors concluded that reticulocytopenia occurring in patients with idiopathic auto-immune hæmolytic anæmia is a serious prognostic sign. Bone-marrow biopsy, performed on each of these patients with reticulocytopenia, was reported to reveal erythroid hyperplasia. In discussing these findings, Crosby and Rappaport suggest that the patients with reticulocytopenia may have suffered from a more severe auto-immune disease than those whose marrow responded to anæmia with a marked reticulocytosis. They consider that the concept of the "reticulocyte pool" in the bone marrow, postulated by Seip (1953), helps to elucidate the mechanism of reticulocytopenia, occurring in acquired hæmolytic anæmia from causes other than marrow aplasia, marrow replacement or acute "aregenerative crisis". This concept is based upon the probability that maturing red cells are not usually released by the bone marrow at the instant when they lose their nuclei, and that young reticulocytes continue to mature for a variable time, providing a reserve of red blood cells during the interval before they are called to active duty. Crosby and Rappaport consider that reticulocytopenia in hæmolytic disease is undoubtedly concerned with the "reticulocyte pool", and that it results from abnormalities of the mechanisms which control the size of the "pool", from injury or destruction of the red cells which comprise it, or from disturbances of the erythroblasts which supply it with young reticulocytes.

In Case VIII of this series, reticulocytopenia persisted for a period of 16 weeks, and bone-marrow biopsy, performed during the tenth week, revealed erythroid hyperplasia. The rate of hæmolysis was markedly increased over this period, and the hæmoglobin value was noted to be less than seven grammes *per centum* on two occasions. The positive result of the direct Coombs test indicated an auto-immune basis for the hæmolytic anæmia. Finally, despite the occurrence of reticulocytopenia in the presence of severe hæmolysis, the reticulocyte count returned to normal levels, the rate of hæmolysis ceased to be excessive, and the child made a complete recovery from her illness.

Acquired Hæmolytic Anæmia following the Ingestion of Naphthalene

Anæmia following the ingestion of naphthalene was first recognized in the middle of the nineteenth century, as a result of the use of this substance as an "intestinal antiseptic" for patients suffering from typhoid fever. More than half a century later, in Brazil, Smillie (1920) observed hæmolytic anæmia in four of 81 patients who had received beta-naphthol for the treatment of hookworm infestation, and postulated an individual hypersensitivity to this drug. Taylor and Russel (1932) noted anæmia, jaundice and hæmoglobinuria in an African Negro who had ingested two mothballs, and differentiated the naphthalene-induced hæmolytic anæmia from blackwater fever.

Zuelzer and Apt (1949) reported the histories of four infants who suffered from acquired hæmolytic anæmia following the accidental ingestion of naphthalene in the form of mothballs, and since this publication at least 10 further cases of this type have been reported amongst American children. Most of the affected children have been Negroes, aged between 18 months and three years, who have usually suffered from a variable degree of fever and some acute gastro-intestinal disturbance within about 24 hours of ingesting the naphthalene. A significant degree of anæmia, frequently associated with hyperbilirubinæmia and transient hæmoglobinuria, was usually apparent about 48 hours after the onset and persisted in most cases for at least five to six days. The anæmia was usually accompanied by spherocytosis and by the presence of fragmented erythrocytes, in which the hæmoglobin

was segregated into one part of the cell, leaving a seemingly empty area with frayed borders.

Diagnosis is not easy unless there is a known history of ingestion of naphthalene in some form. Zuelzer and Apt stressed the importance of specific questioning as to the possibility of ingestion of mothballs. Chusid and Fried (1955) described the case of an infant who developed acute hæmolytic anæmia after ingesting toilet-bowl deodorant cakes, and emphasized the fact that household substances other than mothballs may contain naphthalene; these include certain deodorants, moth-proofing sprays, moth flakes and insecticides. The diagnosis of naphthalene poisoning may be confirmed by the recognition of a naphthalene odour from the urine during the very early stages of the disorder.

The presence of mothballs within reach of the three-year-old infant (Case I of this series) suggests that the hæmolytic anæmia may have resulted from naphthalene poisoning, and stresses the importance of careful inquiry into the possibility of exposure to any product which may contain naphthalene in all cases of acquired hæmolytic anæmia in childhood.

Favism

Faba quidem Pythagorei ubique abstinere.

—Cicero, *De Divinatione*, II, LVIII, 119.

I bid you as did Pythagoras to keep your hands from beans—a harmful food.

—Callimachus of Alexandria.

Approximately 2500 years ago Pythagoras founded a religion, of which the main tenents were the transmigration of souls and the sinfulness of eating beans. The first of the 10 commandments which he issued to his disciples dealt with abstension from beans, and may have been based on some knowledge of the illness which frequently followed the ingestion of beans of the *Vicia fava* type by the people of his Mediterranean world. This illness, known as favism, usually follows the ingestion of raw or partly-cooked beans of the *Vicia fava* type, or the inhalation of the pollen of such beans by a person of Mediterranean origin. As Dacie (1954) has pointed out, the salient features of this disease are the sudden development of an acute episode of hæmolysis, usually followed by jaundice and often accompanied by hæmoglobinuria.

Apparently favism still has a very wide distribution in the Mediterranean area during the summer months, particularly amongst the inhabitants of Sardinia, Italy, Sicily, Greece, Turkey, Cyprus, Malta and some areas of North Africa (Luisada, 1941; Diggle, 1953). The susceptibility to this disorder is familial as well as racial, and children seem more prone to suffer from the severe forms of this disorder than adults. During the past 20 years at least 12 cases of favism have been reported in North America, each of the patients being of Mediterranean descent and most of them Italians.

In addition to P.P. (Case III of this series), two other cases of favism have been reported in this country (Brooks *et alii*, 1958; Moore, 1958). As recent figures obtained from the Commonwealth Department of Demography (personal communication, 1958) suggest that approximately 2·4% of the Australian population are of Mediterranean birth, and as the common Australian broad bean is *Vicia fava*, it seems that favism may possibly be more common in Australia than has been realized.

Glutathione Deficiency of Erythrocytes

Szeinberg *et alii* (1957) suggest that a constitutional anomaly of erythrocytes—namely, glutathione deficiency—is essential for the development of acquired hæmolytic anæmia following ingestion of *Vicia fava*, sulphonamides and para-amino salicylic acid, and observe that "it seems certain that the glutathione deficiency is not a result of hæmolysis, but exists prior to it". They consider that the defect is racially determined, and probably hereditary, in view of the high proportion of persons with glutathione deficiency among the relatives of affected patients.

A significant depression of the glutathione content of the erythrocytes has been demonstrated in Sephardic Jews with a past history of hæmolytic anæmia following the ingestion of *Vicia fava*, sulphonamides and para-amino salicylic acid (Szeinberg *et alii*, 1957), in the relatives of such patients (Szeinberg *et alii*, 1957), in Negroes with a past history of hæmolytic anæmia following the ingestion of naphthalene (Zinkham and Childs, 1957), and in Negroes with a past history of hæmolytic anæmia following the ingestion of "Primaquine" (Beutler *et alii*, 1955).

A determination was made of the glutathione content of the erythrocytes of P.P. (Case III of this series), who is of Greek racial extraction, and is considered to have suffered from favism. The glutathione content of the erythrocytes of this patient was significantly lower than the mean. It is interesting to note that this determination was made more than one year after the patient's recovery from the illness.

Szeinberg and his associates suggest that sensitization to the noxious agent may be

necessary for the development of hæmolytic anæmia following ingestion of *Vicia fava* and sulphonamides, as subjects with glutathione-deficient erythrocytes have ingested *Vicia fava* and sulphonamides without evident harm, and a patient with glutathione-deficient erythrocytes developed hæmolytic anæmia only after a second course of sulphadiazine therapy. Hæmolytic anæmia occurred in each of eight Negroes with glutathione-deficient erythrocytes to whom Beutler *et alii* (1955) administered " Primaquine " ; this suggests that sensitization to the drug is not a factor in the development of hæmolytic anæmia following the administration of " Primaquine ".

Zinkham and Childs (1957) found a glutathione deficiency in the erythrocytes of four Negro patients who developed severe hæmolytic anæmia after exposure to naphthalene mothballs. Included in this group were a mother and her child, who both developed hæmolytic anæmia in the immediate post-partum period, as a result of the nibbling of mothballs by the mother during the last trimester. When erythrocytes of these four patients were incubated with acetylphenylhydrazine, naphthalene products (alpha and beta naphthoquinone and alpha and beta naphthol) and menadione sodium bisulphite, there was a marked fall in the glutathione content of the already deficient erythrocytes. These abnormalities in glutathione metabolism of the erythrocytes of the infant gradually disappeared during the first eight weeks of life, while those of the erythrocytes of the mother persisted.

The families of 17 patients whose erythrocytes were found to be glutathione-deficient were studied by Zinkham and Childs ; of these 17 families, 15 were Negro and two were Greek. The distribution of the subjects with abnormalities of glutathione metabolism in these families indicated that the defect was genetically determined and associated with the presence of a single dominant gene. The incidence of abnormalities in glutathione metabolism in new-born infants was found to be much higher than among the children and adults tested ; this suggests that the defect in new-born infants reflects some metabolic immaturity. Zinkham and Childs consider that glutathione deficiency of erythrocytes is a factor in the development of hæmolytic anæmia following the administration of vitamin K to new-born and premature infants.

While many questions remain unanswered, these studies of the racial, genetic and developmental factors associated with abnormalities of glutathione metabolism of erythrocytes represent a major advance in our understanding of the pathogenesis of some of the acquired hæmolytic anæmias of childhood.

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DAY-TO-DAY VARIATION OF HÆMOGLOBIN VALUE, HÆMATOCRIT READING AND ERYTHROCYTE SEDIMENTATION RATE IN HEALTHY SUBJECTS[1]

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SUMMARY

The effects of menstruation and day-to-day variation of the blood hæmoglobin, hæmatocrit and sedimentation values have been studied. In our series of 13 women, there was no premenstrual fall in the hæmoglobin and hæmatocrit values, although there appeared to be a slight fall in the mean hæmoglobin value during the first four days of the menstrual cycle. The mean values of the Wintrobe and Westergren sedimentation rates were raised during the first four days. The correlations between the hæmatological values were computed. The hæmoglobin value was positively and strongly correlated with the hæmatocrit value. There was good agreement between the results of the two sedimentation methods, and the coefficients of variation were approximately equal. The weight was negatively correlated with the hæmatocrit, Westergren sedimentation and Wintrobe sedimentation values.

COTTER *et alii* (1953) studied the variation in the hæmoglobin values of individuals. They found some differences that were not due to errors in technique, diurnal variations, the external temperature or the amount of exercise before the test. There was no fall in the mean hæmoglobin value in the premenstrual days, but the possibility remained that such a fall did occur in a few women.

The earlier work has been repeated on 13 female subjects, with the object of investigating more thoroughly the effect of premenstrual hydræmia on the hæmoglobin concentration. The weight of the subject was recorded whenever blood samples were collected, and the first day of menstruation was noted. In addition, the day-to-day variation of the hæmoglobin value was measured in six male volunteers. The erythrocyte sedimentation rate was also determined by the methods of Wintrobe and Westergren on every blood sample obtained. It is believed that this is the first occasion on which the day-to-day variation of the sedimentation rate has been investigated in apparently healthy subjects.

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METHODS AND MATERIALS

The volunteers, the females ranging in age from 18 to 40 years and the males from 30 to 55 years, were all members of the staff of the New South Wales Red Cross Blood Transfusion Service. They were engaged in nursing, clerical or laboratory work, but the nature of the work did not vary from day to day. Blood samples were collected from each subject at the same time of the day on the Mondays, Tuesdays, Thursdays and Fridays of four consecutive weeks. The blood was obtained by venepuncture from the veins of the cubital region with a syringe and needle. A tourniquet to compress the veins was applied to the arm for not more than 30 seconds and was removed immediately the needle entered the vein. For the Westergren sedimentation test, 2·0 millilitres of blood were measured from the syringe and mixed with 0·5 millilitre of 3·8% sodium citrate solution in a test tube. The remainder of the blood (approximately 3·0 millilitres) was added to a small tube containing crystals of ammonium and potassium oxalate as previously described (Cotter *et alii*, 1953). This second sample was used for the hæmoglobin, hæmatocrit and Wintrobe sedimentation tests, which were performed by the techniques described by Walsh *et alii* (1953). The Westergren sedimentation result was read at 60 minutes, and the tubes were held in a strictly vertical position in a special stand throughout the period.

RESULTS

The mean values and the average standard deviations of repeated measurements on single subjects are shown in Table I. The variation within subjects has been calculated by a standard statistical procedure, in which the variances of the different subjects are pooled. The standard deviation is the square root of the mean variance.

TABLE I

Results of Tests Performed on Six Male and 13 Female Healthy Subjects

Estimation	Sex	Mean	Standard Deviation of Repeated Measurements on a Single Individual	Coefficient of Variation as Percentage	Degrees of Freedom
Hæmoglobin value (grammes per 100 millilitres of blood)	M. F.	15·11 14·23	0·36 0·39	2·4 2·7	89 192
Hæmatocrit reading (percentage value of packed cells)	M. F.	44·0 40·3	1·08 1·23	2·5 3·1	87 192
Sedimentation rate, Wintrobe (millimetres in one hour)	M. F.	8·73 11·81	2·85 4·10	32·6 34·7	89 192
Sedimentation rate, Westergren (millimetres in one hour)	M. F.	3·81 8·06	1·06 2·34	27·8 29·0	89 192
Weight (kilograms)	F.	56·8	0·48	0·8	192

Hæmoglobin and Hæmatocrit Values

In both sexes the standard deviations of the hæmoglobin values (0·36 and 0·39 respectively for males and females) are slightly lower than the corresponding values obtained in the previous survey (Cotter *et alii*, 1953), but indicate that the day-to-day variation in the values is approximately the same in both sexes. In relation to the mean values, the standard deviations are approximately the same for the hæmoglobin and hæmatocrit values, the coefficients of variation ranging from 2·4% to 3·1% in Table I. It should be noted that Cotter *et alii* (1953) performed essentially the same experiment as that reported in the present paper. They found that the standard deviation of repeated counts on different days was far higher than could be accounted for by experimental error as estimated by Walsh *et alii* (1953).

Sedimentation Rates

The mean values obtained from the Wintrobe sedimentation tests are greater than those found by Walsh *et alii* (1953) in an earlier survey, and in both sexes the standard deviations are large. The Westergren method is not usually employed in this laboratory; but the mean values and the standard deviations were both less than the corresponding values obtained by the Wintrobe method. The mean value of all measurements on a subject was plotted against the standard deviation for the same subject. The results for both methods are shown in Figure I, from which it can be seen that the range of results increases as the mean value increases. There is no difference in this regard between the Wintrobe and Westergren methods. Although the day-to-day variation appears greater by the Wintrobe method, the mean coefficient of variation (standard deviation divided by mean) is approximately the same for both methods (34% for the Wintrobe method and 28% for the Westergren method).

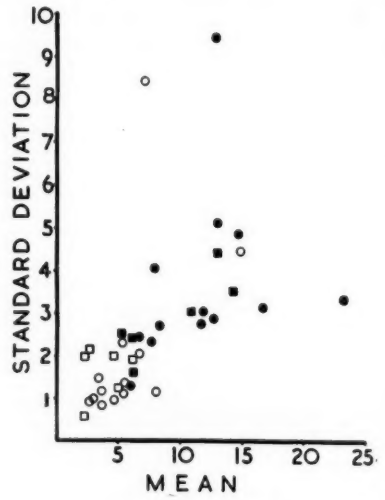

FIGURE I

Relationship between the mean values and the standard deviations of sedimentation rates determined on 13 female and six male subjects. Each point represents the mean value of 16 estimations. Measurements by the Westergren method are represented by open squares and open circles for male and female subjects respectively. In the same way, measurements by the Wintrobe method are represented by solid squares and solid circles

The Menstrual Cycle

Table II shows the mean values of the findings in relation to the menstrual cycle. The number observed on any one day of the cycle is small, but is sufficient to establish a definite pattern. During the four days of menstruation, the day of onset and the next three days, the mean

hæmoglobin value is 13·9 grammes compared with an overall mean of 14·2 grammes, and the mean hæmatocrit value is 40·0% compared with the overall mean of 40·8%. The sedimentation rate, measured by both the Wintrobe and the Westergren methods, is increased during menstruation, and the mean weight is slightly greater during this time than the mean of all weight estimations. If the increased weight during the menses is due to the accumulation of body water, the lower hæmoglobin and hæmatocrit values could be the result of hydræmia. This pattern, however, was not seen in the three days immediately preceding the onset of menstruation, when hydræmia might be also expected. During this time the mean hæmoglobin and hæmatocrit values were little different from the mean values of all estimations.

Associations between Results of Different Tests

The coefficients of correlation between the hæmoglobin value, hæmatocrit reading, sedimentation rate and weight are shown in Table III. The significant correlations between the hæmoglobin and hæmatocrit values are not surprising, because any changes that occur from day to day in the volume of the red cells must be small. Consideration of physical factors suggests that there should be a correlation between the sedimentation rate and the hæmoglobin or hæmatocrit values. This has been demonstrated by Wintrobe and Landsberg (1935), who diluted the patient's blood with his own plasma before the tests were carried out. They were thus able to work with rather gross differences in red cell concentrations. The hæmoglobin values of single subjects under physiological conditions cannot vary so greatly, and we find that we are really correlating small changes in the two variables, somewhat distorted by experimental error. Although most of the coefficients correlating either hæmoglobin value or hæmatocrit reading with either Wintrobe or Westergren sedimentation rates are negative, only two out of the eight have attained significance at the 5% level.

TABLE II

Mean Values of Findings in Relation to Menstrual Cycle

Menstrual Time	Number of Observations	Hæmoglobin Value (Grammes per 100 Millilitres)	Hæmatocrit Reading (Percentage of Column)	Sedimentation Rate (Millimetres in One Hour)		Weight (Kilograms)
				Wintrobe Method	Westergren Method	
−15	5	14·5	40·9	10·6	4·7	54·9
−14	5	14·7	42·0	9·2	4·3	54·7
−13	4	14·0	39·7	16·1	8·2	57·4
−12	5	14·3	40·7	10·0	4·7	56·7
−11	5	14·8	42·2	7·5	2·9	55·7
−10	4	14·8	42·0	14·1	6·2	53·6
−9	5	14·3	40·3	17·2	6·9	56·2
−8	7	14·4	41·9	12·4	6·6	55·1
−7	7	14·3	40·5	13·1	6·4	53·8
−6	5	14·1	39·6	17·1	8·4	56·1
−5	6	14·3	41·4	11·9	6·6	55·9
−4	4	14·5	42·0	10·2	5·0	54·3
−3	4	14·6	40·5	15·0	5·5	54·2
−2	6	14·1	40·8	14·4	7·1	57·0
−1	9	14·1	40·8	11·1	6·6	58·4
0	8	13·8	39·5	11·8	5·9	57·2
1	7	13·9	39·8	15·0	8·2	59·2
2	9	13·9	40·2	14·9	9·0	60·4
3	7	14·0	40·5	14·8	7·7	58·0
4	5	14·4	40·1	13·7	5·5	57·8
5	5	14·6	42·3	10·7	3·6	59·4
6	6	14·6	41·9	9·1	3·5	59·0
7	7	14·2	40·9	10·0	4·9	57·1
8	5	14·4	41·1	10·9	5·1	59·8
9	6	14·2	40·7	9·7	3·5	62·6
10	5	14·2	40·7	9·1	4·2	62·6
11	3	14·1	40·7	13·3	5·7	60·6
12	4	14·9	42·4	9·2	3·5	60·4
13	4	14·3	41·2	8·2	3·4	61·9
14	3	13·7	38·5	10·2	5·0	59·9
Mean all days	208	14·2	40·8	11·8	5·7	57·7
Mean during menstruation	31	13·9	40·0	14·1	7·8	58·8
Mean of three days before onset of menstruation ..	19	14·2	40·7	13·0	6·5	57·0

Significant negative correlations were found in female subjects between weight and hæmatocrit reading and between weight and Wintrobe sedimentation rate.

TABLE III

The Association between Hæmoglobin Value, Hæmatocrit Reading, Sedimentation Rate and Weight

Measurements	Sex.	Coefficient of Correlation	Significance Level
Hæmoglobin value and hæmatocrit reading	M.	0·58	0·001
	F.	0·62	0·001
Hæmoglobin and Westergren sedimentation rate	M.	−0·13	Not significant
	F.	−0·14	Not significant
Hæmoglobin and Wintrobe sedimentation rate	M.	0·12	Not significant
	F.	−0·02	Not significant
Hæmatocrit reading and Westergren sedimentation rate	M.	−0·23	0·05
	F.	−0·14	Not significant
Hæmatocrit and Wintrobe sedimentation rate	M.	−0·13	Not significant
	F.	−0·24	0·002
Wintrobe and Westergren sedimentation rates	M.	0·30	0·01
	F.	0·70	0·001
Weight and hæmoglobin value	F.	−0·12	Not significant
Weight and hæmatocrit reading	F.	−0·23	0·01
Weight and Westergren sedimentation rate	F.	−0·17	0·05
Weight and Wintrobe sedimentation rate	F.	−0·28	0·001

DISCUSSION

Variations in the hæmoglobin and hæmatocrit values from day to day could be the result either of changes in the plasma volume or of changes in the relative amounts of red cells and plasma in the vessels of different parts of the body. These variations occur in the same direction and are of the same order of magnitude for both tests and in both sexes. Sometimes they are as great as $\pm 8{\cdot}5\%$ of the mean values. To date there is no information as to whether similar changes occur in patients confined to bed or in anæmic subjects prior to treament.

The sedimentation rate value also shows day-to-day variations of considerable magnitude, and these changes are independent of those which occur in the hæmoglobin and hæmatocrit values. Furthermore, the fluctuations are not dependent on the method used for measuring the values. The fluctuations are less by the Westergren than by the Wintrobe method, but the mean values are also less, and it was shown that the range of values in an individual is proportional to the mean value. Walsh *et alii* (1953) also demonstrated that the standard errors of measurement, which include all experimental errors, are related to the mean value. The standard deviations in the present series are, however, much greater than those observed when replicate measurements were made on the same samples of blood with comparable mean sedimentation values. The day-to-day variations are therefore due to physiological factors rather than to experimental errors, and are probably the result of variations from day to day in the relative proportions of the various plasma proteins. No attempt has been made in this work to assess the existence or significance of variations in the concentration of these proteins.

No significant fall occurred in the hæmoglobin or hæmatocrit value of females immediately before or during the menstrual period. The slight increase in body weight during the period may have been caused by an increase in the water content of the body; but any increase in the plasma volume produced little change in the concentration of the cellular elements of the blood. On the other hand, an increase was observed in the sedimentation rates before and during menstruation, and this was probably caused by changes in the plasma protein pattern at this time.

The results reported in this paper show that the Wintrobe and Westergren methods both detect day-to-day variations to an equal extent in relation to the mean values. Changes due to disease processes have not been investigated; but several workers (Davis, 1946; Gilmour and Sykes, 1951; Goldberg, Glynn and Bywaters, 1952; Goldberg and Conway, 1952) believe that the Westergren method is more sensitive as a pointer to organic disease.

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THE ROLE OF BODY FLUID VOLUME AND PLASMA "ACTIVE" OSMOTIC PRESSURE IN THE CONTROL OF URINE FLOW AND FLUID INTAKE IN MAN[1, 4]

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SUMMARY

Observations of body weight, plasma osmotic pressure, daily urine flow and daily fluid intake in a patient recovering from an œdematous state indicate that changes in both plasma osmotic pressure and body fluid volume have an influence on the volume of urine formed, and possibly on the volume of fluid drunk.

The control system acts in such a way that a rise of plasma osmotic pressure produces a fall in urine flow and a rise in body fluid volume produces an increase in urine flow.

A method is described which enables plasma freezing point to be determined on one millilitre of plasma.

IN previous discussions (Lowe, 1957) on data obtained from a large series of patients suffering from abnormal states of body fluid volume control, it was concluded that the observed relationships between urine flow and body weight indicated that two facets of body fluid were controlling agents. One of these appears to be the volume of some portion of the vascular volume, and it was suggested that the other might be the osmotic pressure of some portion of the body fluid.

In a number of patients recovering from œdema, the osmotic pressure of the plasma was determined on a number of occasions. Although these osmotic pressure determinations referred to the time of sampling only and the urine flow estimations to 24-hour periods, it appears that a linear relationship between plasma osmotic pressure and urine flow can be postulated.

Owing to the need for frequent estimations, a method of determining plasma osmotic pressure on small quantities was desired, and this method is described in this paper.

When the possibility that the body fluid could be considered as an "open" storage system was under discussion (Lowe, 1956*a*), it was suggested that the intake of fluid, demanded by thirst and salt hunger, should be related to body fluid volume and plasma osmotic pressure in a manner the inverse of that seen in the control of urine flow.

The data obtained from a patient in whom both plasma osmotic pressure and body fluid volume were continuously varying indicate the relationships of inflow and outflow of fluid to body fluid volume and plasma osmotic pressure.

METHOD

Urine flow, fluid intake and body weight were determined under the specific conditions used in previous observations (Lowe, 1951). These included a diet of fixed caloric value, sodium content and food type.

Plasma osmotic pressure was measured and recorded as the depression of the freezing point of the plasma below that of water. (Depression of freezing point by 0·001° C. is equivalent to a rise in osmotic pressure of 0·5 milliosmol per litre.) Venous blood was drawn into a dry syringe, rapidly transferred to a tube containing dried heparin and then centrifuged. One millilitre of the plasma was taken and its freezing point determined. For each sample the contents of glucose and urea were determined, and the contribution of each of these to the depression of the freezing point was read from previously determined tables and subtracted from the observed freezing point depression. This correction was considered necessary, as Verney (1947) has shown these two substances to have little or no action on the osmoreceptors of the body. All freezing point depressions quoted refer to this corrected value.

The freezing temperature of the plasma was determined with a small thermistor (F2200 Stantel), the resistance of which was measured by a wheatstone bridge with a 450 ohms galvanometer as a null-point indicator. The relationship between thermistor resistance and temperature was determined by measuring the resistance of the thermistor at the freezing temperatures of a series of accurately prepared sodium chloride solutions, the freezing points of which were calculated from International Critical Tables (Hall and Sherill, 1928). The concentrations of these standard solutions were chosen so that the appropriate range of temperatures was covered. The relationship was found to be essentially linear over the small range of temperatures needed.

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The freezing chamber consisted of a narrow glass test tube, which was closed with a cork through which passed the thermistor and a thermally insulated stirring wire. The freezing bath was a solution of ethylene glycol in water, contained in an insulated tank and cooled by a refrigerator unit to —3° C. Also suspended in the freezing bath was a test tube, of sufficient diameter to contain the freezing chamber, which could be held by cork rings so that an air space separated the two tubes. This tube is referred to as " the holding tube ".

To determine the freezing point of either plasma or standard solution, one millilitre was introduced into the freezing chamber. The sample was gently and continuously stirred by hand, whilst an assistant followed, with the bridge, the rise of thermistor resistance. At the moment of freezing there was an abrupt reversal of the steady rise of resistance, clearly indicated by a swing of the galvanometer spot. The freezing chamber was then rapidly placed in the holding tube, where the air jacket prevented fast cooling. This gave ample time to measure accurately the resistance of the thermistor at the freezing temperature.

Reference to the calibration chart gave only the approximate temperature for this resistance, for thermistors are liable to sudden small changes in resistance/temperature characteristics. Measurements of standard solutions bracketing the unknown in freezing temperature were then made. In all cases, duplicate samples of plasma were bracketed by standard solutions, and the duplicate values for freezing temperature were required to differ by less than 0·005° C.; an average of the two values was then taken. By the use of this technique, it is possible to measure the freezing temperature of the solutions to the nearest 0·005° C. with confidence, and to estimate to the nearest 0·001° C.

OBSERVATIONS

Corrected plasma freezing point depression, used as a measure of active plasma osmotic pressure, was determined on many occasions on each of 12 patients suffering variously from œdema due to cardiac failure, nephritis, nephrotic syndrome and endocrine disturbances.

Values of daily urine flow, daily fluid intake, body weight and corrected plasma freezing point depression were plotted, both on three-dimensional models and on two-dimensional graphs. In general the results indicated that much of the scatter of points about a linear regression line relating daily urine flow to body weight was associated with variation in freezing point of the plasma.

The results in Case I (Figures I and II and Table I) may be used to indicate the relationships seen.

CASE I.—This woman, aged 49 years, was suffering from Addison's disease and had mild generalized œdema. During a period of rest in bed she lost some three kilograms in weight. On the fourteenth day, oral administration of delta-hydrocortisone was commenced (five milligrammes three times a day). Within 24 hours diuresis occurred, and her fluid balance became stabilized after the loss of another three kilograms in weight. After an interval of 11 days on a free diet, during which a further weight loss occurred, she was readmitted to hospital to establish more adequately the dosage of hormone required. During this second admission she gained four kilograms in weight.

Table I records the daily urine flow, daily fluid intake and daily body weight, the measurements of the corrected plasma freezing point depression (to the nearest 0·001° C.), and the hormone dosage, throughout both admissions.

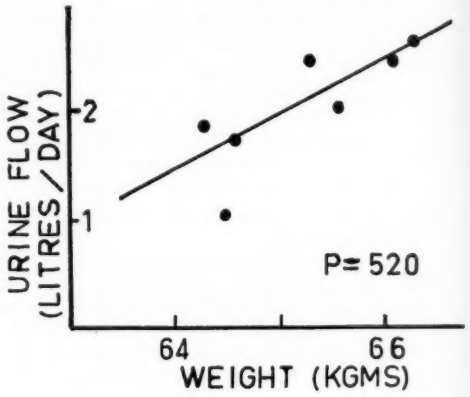

FIGURE I

Graph showing the relationship between rate of urine flow and body weight when the values of rate of urine flow are corrected to a common plasma freezing point depression of 0·520°C

(1) Urine Flow. A regression equation was calculated from the observations made on days 2 to 13 inclusive of the first admission, for which values of urine flow, body weight and plasma freezing point depression were available. This equation is

$$U = -12{\cdot}7 + 0{\cdot}49W - 0{\cdot}033P \quad \ldots\ldots\ldots \text{(I)}$$

where U = urine flow (litres per day), W = body weight (kilograms) and P = corrected plasma freezing point depression (unit = 0·001° C.). It has a correlation

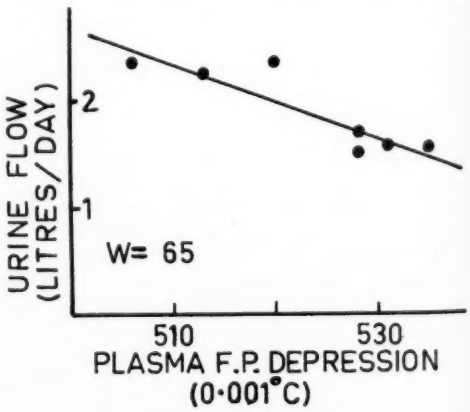

FIGURE II

Graph showing the relationship between rate of urine flow and plasma freezing point depression when the values of rate of urine flow are corrected to a common body weight of 65 kgms

coefficient of 0·85, and $t=3·6$, which lies between the 1% and 5% levels of significance. The t values of the individual regression coefficients also lie between these levels. This equation relating three variables can be represented only by a three-dimensional model, and a projection of the model on a plane diagram may be confusing. For clarity of presentation Figure I shows the relationship between urine flow and body weight when all values of urine flow have been corrected to an arbitrary $P=520$ in accordance with equation I. In Figure II, the relationship between urine flow and plasma freezing point depression for an arbitrary $W=65$ are shown.

For days 15 to 19 of this admission the regression equation is

$$U=-11·8+0·49W-0·033P \quad \ldots\ldots \text{(II)}$$

For the second admission the equation is

$$U=-9·8+0·49W-0·033P \quad \ldots\ldots \text{(III)}$$

It will be noted that the only change in the relationship between urine flow, body weight and plasma osmotic pressure in the different phases of this patient's illness is in the numerical value of the constant. This changed suddenly between days 13 and 15 of the first admission, corresponding in time with the exhibition of delta-hydrocortisone. Another change occurred in the period between the two admissions.

(2) Fluid Intake. Examination of the data concerning fluid intake shows that for days 11 to 21 inclusive of the second admission, during which therapy remained qualitatively the same, the following equation relates inflow, body weight and plasma freezing point depression.

$$I=-7·6-0·15W+0·034P \quad \ldots\ldots \text{(IV)}$$

where I=fluid intake (litres/day), and W and P are as previously defined. Although this has a correlation coefficient of 0·60, and $t=2·5$, which lies between the 1% and 5% levels of significance, the t values of the individual regression coefficients lie above the 5% level. In other periods of observation no definite correlation could be detected.

DISCUSSION

It has been demonstrated in patients recovering from œdema (Lowe, 1957) that daily urine flow has a linear relationship to body weight and hence to plasma volume (Lowe, 1958). In all cases, however, the scatter of points about a regression line relating urine flow and body weight is great, and unless the range of body weight values is large, the scatter obscures this relationship. A possible explanation of this scatter is that there exists at least one other factor which has a control over urine flow of magnitude similar to that of a weight change of the order of one or two kilograms. On the basis of observations (Gilman, 1937; Holmes and Gregersen, 1947; Verney, 1947) that plasma osmotic pressure influences urine flow and fluid intake, it was considered (Lowe, 1956*a*) that this second factor might be plasma osmotic pressure.

The data obtained from this patient suggest that plasma osmotic pressure may in fact

TABLE I

Day	Urine Flow (Litres per Day)	Fluid Intake (Litres per Day)	Weight (Kilograms)	Depression Plasma Freezing Point (°C.)	Hormones		
					Delta-Hydro-cortisone	Hydrocortisone	Fluoro-hydrocortisone
First admission:							
1	2·0	2·1	66·7	0·507			
2	2·1	2·5	66·1	0·531			
3	2·2	2·4	66·3	0·535			
4	1·0	2·4	66·4	—			
5	2·3	2·3	66·0	—			
6	1·5	1·6	64·6	0·528			
7	1·8	2·3	65·6	0·528			
8	—	3·1	65·9	0·532			
9	2·5	2·6	65·3	0·520			
10	1·9	0·9	64·3	0·513			
11	1·5	2·7	65·0	—			
12	1·8	2·4	64·6	—			
13	2·1	2·9	64·5	0·506			
14	1·4	1·7	64·3	0·491	15 mg.		
15	1·9	1·5	62·4	0·490	15 mg.		
16	1·6	1·6	62·3	0·521	15 mg.		
17	1·2	1·3	61·5	0·526	15 mg.		
18	1·4	1·9	61·5	—	15 mg.		
19	1·4	1·9	61·4	0·506	15 mg.		
Second admission:							
2	2·4	1·9	57·2	0·487	10 mg.		0·5 mg.
3	2·2	2·5	57·3	—	10 mg.		0·5 mg.
4	1·9	2·3	57·7	0·520	10 mg.		0·5 mg.
5	1·9	1·8	58·0	0·506	10 mg.	100 mg. intravenously	0·5 mg.
6	1·3	1·7	57·9	0·503	10 mg.		0·5 mg.
7	1·5	1·2	58·1	0·525	10 mg.		0·5 mg.
8	1·8	1·6	58·0	0·530	10 mg.		0·5 mg.
9	1·7	1·4	58·5	—	2·5 mg.	20 mg.	0·25 mg.
10	2·2	1·9	58·4	—		40 mg.	
11	2·0	2·1	58·5	0·520		40 mg.	0·5 mg.
12	2·3	2·1	58·3	0·526		40 mg.	0·5 mg.
13	1·5	1·2	59·1	0·530		20 mg.	0·25 mg.
14	1·8	0·7	59·2	0·531		20 mg.	0·25 mg.
15	1·8	1·2	59·2	0·535		40 mg.	0·5 mg.
16	1·9	2·5	59·9	—		50 mg.	0·5 mg.
17	2·5	2·4	60·2	—		50 mg.	0·5 mg.
18	1·8	1·7	60·5	0·540		50 mg.	0·5 mg.
19	2·3	2·1	60·8	0·547		50 mg.	0·5 mg.
20	1·7	1·4	60·9	0·535		50 mg.	0·5 mg.
21	2·4	2·3	61·2	0·560		50 mg.	0·5 mg.

regulate urine flow in such a way that a rise of osmotic pressure decreases urine flow, and vice versa. Further, the data indicate that this effect is of similar magnitude to that produced by a change in body fluid volume as reflected in the body weight. A change of one kilogram (approximately 1·5% of body weight) or of 0·015° C. (approximately 3% of plasma osmotic pressure) produces a change of 0·5 litre in the daily urine flow.

The data indicate that, when the effects of weight and osmotic pressure have been accounted for, there is, in this instance, no other major factor influencing urine flow (Figures I and II).

The existence of these two controls of similar importance implies that, when urine flow is plotted against body weight, a relationship will be apparent only when the osmotic pressure is constant over the period of observation, or when the weight change is relatively much greater than any osmotic pressure change. The relationship between urine flow and body weight, therefore, is easily demonstrable in patients recovering from considerable œdema, but not readily apparent when weight changes are small. The change in value of the constant in the regression equations in this case is similar to that noted in other cases (Lowe, 1956*b*) occurring either spontaneously or as a result of therapy. In this instance it is presumably due to the administration of hormones, which also produced the few anomalous results.

On general physiological grounds, it would seem probable that these controls are part of the homœostatic mechanisms of the body. The control by plasma osmotic pressure probably operates through the osmoreceptors and the antidiuretic hormone of the pituitary gland. The pathway by which body fluid volume controls urine flow is, however, still obscure.

In previous discussions on the control of body fluid volume, beyond indicating the theoretical possibility that the intake of fluid may be controlled by factors similar to those influencing outflow, no consideration has been given to data relating intake of fluid with body weight or plasma osmotic pressure. For a great many of the patients observed no data are available, because the fluid intake was fixed at a constant arbitrary level. For those who were permitted a self-determined intake, no unequivocal correlations between inflow and body weight were apparent.

However, the data from the present patient show, for a selected period of the second admission, that the intake of fluid may be correlated with the fluid volume of the body and the plasma osmotic pressure (equation IV). If this instance depicts a causal relationship, then the usual lack of unequivocal correlation indicates that there must be other factors which also influence inflow, and these factors have not been made a constant error by the conditions of observation used. This situation would mask any relationship between intake and body fluid volume and plasma osmotic pressure, unless by chance these unknown factors are constant throughout a period of observation, as has apparently happened for the selected period in this case. The existence of these additional factors is to be expected by the participation of fluid intake in at least two of the body's regulating mechanisms—control of fluid volume and control of body temperature. Whilst the conditions specified for observation (Lowe, 1951) minimize daily variation in the temperature control system, it is unlikely that variation was completely eliminated.

It may be concluded that the data presented are consistent with the observations of others that plasma osmotic pressure and body fluid volume influence the output of urine. This influence is such that a rise of plasma osmotic pressure or a fall in body fluid volume produces a fall in the rate of urine formation. These relationships are approximately linear and of similar order.

It is also possible that the same factors have an influence on the intake of fluid the inverse of that on outflow.

These conclusions are based, as are the previous conclusions in this study, on correlations between measurements, and must therefore be considered as tentative. They should, however, prove a stimulus to further study of volume control in terms of the physics of open systems, the implications of which it has not yet been possible to refute.

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CATION STUDIES DURING INTRAVENOUS ANÆSTHESIA[1]

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SUMMARY

The effect of pentobarbitone given intravenously on the hæmatocrit, on the serum sodium and potassium contents, and on the intracellular water, sodium and potassium concentrations of skeletal muscle has been investigated in sheep. $S^{35}O_4$ has been used to determine the extracellular space of muscle and the extracellular fluid volume of distribution.

A significant fall in serum potassium level (from 5·5 to 4·6 milliequivalents per litre) and in hæmatocrit (from 37·4% to 30·2%) occurred under this anæsthetic agent.

There was no change in intracellular water, sodium or potassium concentrations in the extracellular space of skeletal muscle, or in the volume or rate of distribution of $S^{35}O_4$ during anæsthesia.

These results were not altered by splenectomy. The administration of adrenaline produced no changes in the hæmatocrit or in the serum potassium level.

IT is well recognized that normally functioning body cells have the ability to concentrate certain ions within them and to expel others, thus establishing an intracellular-extracellular concentration gradient. Not only has each body cell the ability to do this, but its very existence depends upon it (Ussing, 1953). The maintenance of this state of dynamic equilibrium (Dean, 1941) is dependent upon the utilization of cell energy (McDowall *et alii*, 1955; Hercus *et alii*, 1955). Interference with the energy production within the cell will affect it in various ways; sometimes the changes are reversible and sometimes irreversible.

It is known that changes in serum potassium level occur in intravenous anæsthesia (Goodman and Gilman, 1955). Hæmatocrit changes have also been observed by Bollman *et alii* (1938), Bourne *et alii* (1930) and Graca and Garst (1957). It was decided to investigate these changes further, by studying in skeletal muscle the intracellular sodium, potassium and water concentrations in relation to the serum sodium and potassium and hæmatocrit changes resulting from intravenous sodium pentobarbitone anæsthesia, while ensuring adequate oxygenation of the unconscious animal, and adequate removal of carbon dioxide, thus preventing any gaseous acidosis.

It was also decided to include splenectomized animals in the investigation, as formed elements are alleged to be sequestrated in the spleen and released into the circulating blood when required. Such a mechanism could be reflected by changes in the hæmatocrit.

MATERIAL AND METHODS

All sheep were acclimatized for some weeks in their pens and well established on a constant diet, but were starved for 12 hours before investigations were made. Ewes were used in preference to rams, as urine could be more easily collected by catheter.

Group A

Samples of blood and of muscle from the leg were taken under local anæsthesia, considerable care being exercised to infiltrate only the subcutaneous tissues and not to inject any anæsthetic into the muscle. Animals were then anæsthetized with sodium pentobarbitone given intravenously, the larynx was intubated, and the lungs were ventilated with oxygen by means of a Starling pump respirator and a carbon dioxide to-and-fro absorber. Further samples of blood and muscle were taken after five minutes and one and a half hours, and then the anæsthetic was stopped.

On the following day, further samples of blood and muscle were taken under local anæsthesia. Blood was collected from the jugular vein by means of a 14 gauge needle (to minimize hæmolysis), in amounts sufficient for serum sodium and potassium estimations, and an additional five millilitres were collected in tubes dried after the addition of 0·5 millilitre of a potassium-ammonium oxalate mixture for the determination of hæmatocrit.

Sufficient muscle was taken for at least six samples, each weighing about 60 to 80 milligrammes. These samples were rapidly and carefully blotted and then placed in sealed weighing tubes.

The results obtained from this group enabled us to calculate all the changes in muscle and serum (see Tables I and III), but not the intracellular concentration of cations or water.

[1] Received on July 17, 1958.

[2] Anæsthetist. This work was made possible by a grant from The Royal Australasian College of Physicians.

[3] Science graduate assistant.

TABLE I

Effect of Intravenous Sodium Pentobarbitone Anæsthesia on Serum Sodium and Potassium Levels and on the Hæmatocrit

Group and Number of Sheep	Serum Sodium Level (mEq./l.)		Serum Potassium Level (mEq./l.)		Hæmatocrit (Percentage)	
	Before Anæsthesia	During Anæsthesia	Before Anæsthesia	During Anæsthesia	Before Anæsthesia	During Anæsthesia
Group A: 7	124	125	5·65	4·5	34·0	29·1
Group B: 7	143	137	5·4	4·7	38·8	31·4
Group C: 4	137	135	5·7	4·8	37·2	31·2
Group D: 3	146	154	5·8	4·3	39·1	34·6
Mean of Groups A and B[1]	134±3·2	132±4·1	5·6±0·1	4·6±0·15	36·4±1·6	30·7±1·4
	No difference		$p<0.001$		$p<0.001$	

[1] ±=standard error of mean.

Group B

Animals were prepared as described above and the bladder was catheterized. A carrier-free solution of $S^{35}O_4$ (five microcuries per kilogram) was injected into the jugular vein, and blood samples were taken at frequent intervals up to two hours for estimation of $S^{35}O_4$ content. Urine was also collected at various intervals. Blood and muscle samples were taken under local anæsthesia at 25 minutes for estimation of the following: $S^{35}O_4$ level in serum and in muscle, hæmatocrit, serum sodium and potassium levels, and muscle water, sodium and potassium concentrations. Muscle samples of 300 to 500 milligrammes were used for calculating $S^{35}O_4$ content.

TABLE II

Effect of Intravenous Sodium Pentobarbitone Anæsthesia on Sulphate Space of Muscle and Volume of Distribution

Group and Number of Sheep	Skeletal Muscle Space (ml./100 Gm. Muscle)		Volume of Distribution (ml./100 Gm. Body Weight)	
	Before Anæsthesia	During Anæsthesia	Before Anæsthesia	During Anæsthesia
Group D: 3 ..	14·3±1·8	12·2±1·6	27·1±1·4	32·5±2·8
Group B: 6 ..	13·6±1·4	12·7±1·6	28·4±1·0	36·5±3·1
	No significant difference		$0.02<p<0.05$	

After at least seven days these animals were subjected to the same procedure as before, but were first anæsthetized with sodium pentobarbitone given intravenously and then managed as in group A.

The results obtained from this group permitted calculations of the extracellular space of muscle before and after anæsthesia (Table II). They also confirmed the results obtained in group A.

The two separate estimations before and after the induction of anæsthesia were made at wide intervals, because this simplified the calculations that would have been necessary had we made one injection of $S^{35}O_4$ before anæsthesia, and another of $S^{35}O_4$ immediately after the induction of anæsthesia. No $S^{35}O_4$ can be detected in the sheep five days after injection. Thus, after an interval of a week or more there is no need to take the previous injection into account when calculating the $S^{35}O_4$ concentration.

Group C

Four animals were subjected to the same investigations as in group A, but were not intubated or connected to a Starling pump. Instead they were allowed to breathe air normally during the course of the anæsthetic.

Group D

Similar experiments were conducted on animals that had been splenectomized some six to eight weeks previously.

TABLE III

Effect of Intravenous Sodium Pentobarbitone Anæsthesia on Muscle Water and Muscle Sodium and Potassium Concentrations

Group and Number of Sheep	Muscle Water (ml./1000 Gm.)		Muscle Sodium (mEq./1000 Gm.)		Muscle Potassium (mEq./1000 Gm.)		Sodium (mEq./l. Fibre Water)		Potassium (mEq./l. Fibre Water)	
	Before Anæsthesia	During Anæsthesia	Before Anæsthesia	During Anæsthesia	Before Anæsthesia	During Anæsthesia	Before Anæsthesia	During Anæsthesia	Before Anæsthesia	During Anæsthesia
Group A: 7	780	783	43·4	43·6	89·1	94·2	—	—	—	—
Group B: 7	772	782	39·1	39·5	97·3	95·5	35·4	36·4	144	144
Group C: 4	774	777	39·6	38·4	95·6	97·5	—	—	—	—
Group D: 3	768	779	44·4	48·4	93·1	88·4	36·2	45·1	147	134
Mean of Groups A and B	776 ±6	784 ±8	41·2 ±2·2	41·5 ±1·9	93·2 ±2·1	94·8 ±1·4	35·4 ±2·1	36·4 ±1·8	144 ±3·1	144 ±2·7

Group E

The effect of adrenaline on the hæmatocrit and on the serum potassium level was determined by injecting one or two millilitres of adrenaline tartrate (1 : 1000 solution) and collecting blood samples at various intervals up to half an hour.

Methods

Sodium and potassium in both serum and muscle were estimated by the E.E.L. flame photometer, the serum being suitably diluted, and the muscle being first dissolved in nitric acid (50%) and then diluted in water.

Hæmatocrits were read after centrifugation of the oxalated blood samples in Wintrobe tubes at 3000 revolutions per minute for 30 minutes. Muscle-water was calculated from the difference in weight of samples of muscle when fresh and after being dried in an oven at 120° C. for 24 hours.

$S^{35}O_4$ in serum, muscle and urine was precipitated as benzidine sulphate as described by Sheatz and Wilde (1950) and by Walser (1952), sufficient sodium sulphate having been added as a carrier to give a precipitate of finite thickness. The precipitate was filtered through a stainless steel demountable filter cup (Tracer Elements Report No. 1, 1951), collected on number 42 filter paper, mounted on a flat metal disposable disk and counted under a thin mica end-window counter of 20th Century Electronics type EW.3H (Henriques *et alii*, 1946). Similarly treated aliquots were used for reference counting. The determinations of $S^{35}O_4$ in the serum and muscle samples were respectively expressed as volume of distribution of $S^{35}O_4$ per 100 grammes of body weight and as millilitres per 100 grammes of muscle.

After allowance for urine excretion and correction for plasma water and Donnan effect had been made, the extracellular sodium concentration (EcNa) was calculated as described by Leaf *et alii* (1954).

Intracellular concentrations of sodium and potassium were calculated and expressed as milliequivalents per litre of fibre water. For example :

$$\text{Na (m.Eq/l. fibre water)} = \frac{(\text{Total Na}-\text{EcNa})\ \text{m.Eq/1000 G muscle} \times 1000}{(\text{Total } H_2O-\text{Ec}H_2O)\ \text{ml. per 1000 G muscle}}$$

where EcNa = extracellular space × extracellular sodium concentration and EcH_2O = extracellular water.

The amount of blood in muscle samples was not significant, being less than 0·5% of the total volume of muscle. This was estimated by soaking heparinized muscle samples in saline, counting the red cells thereby extracted and relating this finding to the animal's whole blood count.

RESULTS

Serum Electrolytes

The serum sodium level showed no change ; yet there was a significant fall in serum potassium level, which fell immediately after the induction of anæsthesia and kept on falling slowly during the course of the anæsthesia, but returned nearly to normal in 24 hours (see Table I and Figure I).

Hæmatocrit

The hæmatocrit followed a course similar to that of the serum potassium level, as shown in Table I and Figure I.

Sulphate

$S^{35}O_4$ studies showed an apparent but insignificant increase in the volume of distribution of $S^{35}O_4$ during anæsthesia ($0{\cdot}02 < p < 0{\cdot}05$; Table II).

There was no change in the sulphate space of skeletal muscle (Table II).

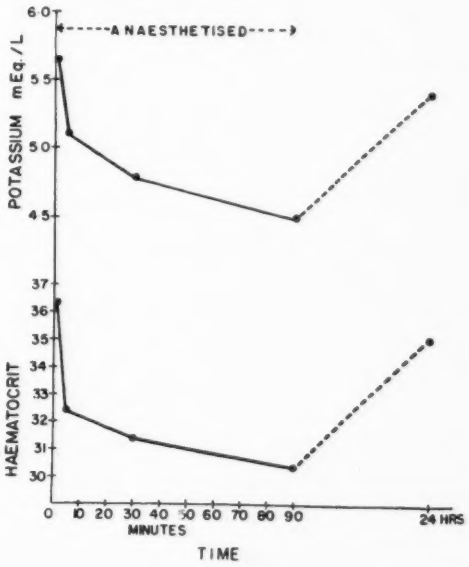

FIGURE I

Changes in serum potassium level and hæmatocrit during anæsthesia

There was no significant difference between conscious, anæsthetized or splenectomized sheep either in the volume of distribution or in the rate of equilibration of $S^{35}O_4$ throughout the body. The rate of renal excretion of $S^{35}O_4$ did not vary significantly from normal, under anæsthesia.

Muscle

No changes were found in the muscle water or the muscle electrolytes, sodium and potassium, either in the whole sample, expressed as milliequivalents per 1000 grammes of muscle, or in the concentration of these cations within the muscle fibres expressed as milliequivalents per litre of fibre water (Table III).

The reproducibility of results is shown in Figure II, which illustrates the volume of

distribution of $S^{35}O_4$ in the same animal over three weeks at weekly intervals. There was a slight overall fall in the hæmatocrit and the serum potassium level, probably due to incomplete restoration of blood volume, since during a full investigation of this nature some 10% of the animal's blood volume may be withdrawn in the repeated venepunctures.

Group C

In group C (Tables I, II and III) the animals, which were not intubated and not mechanically ventilated with oxygen, but allowed to breathe air normally during the course of their anæsthesia, gave results which were no different from those in group A.

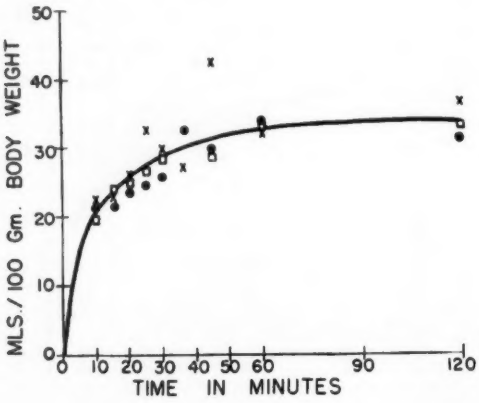

FIGURE II

Volume of distribution of $S^{35}O_4$ on three successive weeks; solid circles, first week; crosses, second week; open squares, third week

Group D

The results for group D are shown in Tables I, II and III. The spleen did not appear to play any significant part in these experiments, as the same results were obtained in both normal and splenectomized animals.

Group E

Adrenaline tartrate injected intramuscularly produced no alteration in the hæmatocrit or in the serum potassium level of the sheep in group E.

Comment

All groups of sheep gave similar results; minor differences noted between the groups may be due to the fact that the sheep were of different breeds, or that they were purchased from different sources, and varied somewhat in age and physical condition.

DISCUSSION

Intravenous anæsthesia with sodium pentobarbitone in sheep causes a significant fall in serum potassium level and in the hæmatocrit. These effects are observed within five minutes of administering the anæsthetic agent, and become slowly more pronounced as the anæsthesia continues; but within 24 hours they have virtually returned to normal. There is no corresponding fall in serum sodium level; this leads to the assumption that there is no shift of water from skeletal muscle to cause any possible hæmodilution, as such "cell water" is virtually sodium-free. On the other hand, owing to the large difference between serum potassium and intracellular potassium concentration (the ratio is 5·6 : 144), a relatively large amount of potassium could go from the serum into the cells without its being possible to detect any such shift.

Under barbiturate anæsthesia the spleen may become dilated (Goodman and Gilman, 1955), and formed elements may be sequestrated in it; but in the sheep studied here it was considered that the spleen did not play an important role, as the same results were obtained in splenectomized animals. Bollman *et alii* (1938) showed that barbiturates caused a rise in plasma volume with a fall in the hæmatocrit—these were not affected after splenectomy; also Graca and Garst (1957) did not think that the changes in hæmatocrit were attributable to changes in splenic volume. Whether or not there are other avenues of sequestration is not evident in these experiments.

The importance of adrenaline in affecting electrolyte balance has been demonstrated by various workers. Adrenaline produces a rise in serum potassium level in cats (D'Silva, 1933, 1934; Robertson and Peyser, 1951; Muirhead *et alii*, 1954), but a fall in dogs (Rogoff *et alii*, 1950; Jacobson *et alii*, 1951; Houssay *et alii*, 1937; Goffart and Perry, 1951). In the sheep studied here, however, injection of adrenaline produced no alteration in the serum potassium level.

Acid-base disturbances have been shown to play an important part in electrolyte balance (Harris and Maizels, 1951; Darrow, 1946; Cotlove *et alii*, 1951). It has been shown that dogs anæsthetized with sodium pentobarbital and exposed to an atmosphere of 30% to 40% carbon dioxide in oxygen for three to five hours develop an arterial blood pH of 6·7, and the plasma potassium level rises from 3·9

to 6·9 milliequivalents per litre (Sealy *et alii*, 1954).

The dangers of carbon dioxide retention have also been emphasized by Dripps and Severinghaus (1955), who pointed out that during many types of general anæsthesia alveolar carbon dioxide concentration can vary from a normal 5·6% to as high as 30%.

In the present experiments it is seen that the hæmatocrit and serum potassium changes occurred within five minutes of the induction of anæsthesia. During intubation it is possible that gaseous acidosis may develop; but after the animal had been ventilated by manual squeezing of the oxygen bag, and then mechanically ventilated by the Starling pump with the carbon dioxide absorbed by soda lime, there was no evidence of a gaseous acidosis; in fact, there were often indications of a mild "washing out" of carbon dioxide, as the animal was sluggish in recovering its own respiratory rhythm after disconnection from the pump. At this stage such animals always returned to normal breathing when given 5% carbon dioxide and 95% oxygen much more quickly than if given 100% oxygen.

The animals (group C) which were allowed to breathe air without any mechanical ventilation during anæsthesia could theoretically have developed mild gaseous acidosis; but this could not have influenced the results, which showed no difference from those obtained in other groups. It is therefore unlikely that, under the conditions of this experiment, any gaseous acidosis could develop, sufficient to play any part in the hæmatocrit or electrolyte changes observed.

It has been suggested that, under barbiturate anæsthesia, the plasma volume rises (Bollman *et alii*, 1938; Goodman and Gilman, 1955) owing to a shift of fluid from the tissue spaces into the blood-stream. If this occurred, we should expect a fall in the hæmatocrit, but not necessarily any fall in plasma potassium level.

Although sodium pentobarbitone may affect cell metabolism (Toman, 1952; Quastel, 1939; Holland, 1954), the site or nature of such action does not affect the water, sodium or potassium exchanges in skeletal muscle.

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CATION STUDIES DURING EXPERIMENTAL HYPOTHERMIA[1]

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SUMMARY

Experimental hypothermia in sheep was produced by immersing them in an iced water bath, the body temperature being lowered to 28°–30° C. The animal was anæsthetized with sodium pentobarbitone given intravenously and mechanically ventilated with oxygen during the procedure.

There was a significant fall in the serum potassium level and a rise in the hæmatocrit as a result of this cooling. Examination of specimens of skeletal muscle showed that water and sodium had entered the muscle fibre, with a consequent dilution of its potassium. The sulphate space of skeletal muscle was reduced.

The changes in muscle suggest either an anoxic effect possibly resulting from the hæmodynamic changes (lowered cardiac output, increased blood viscosity, lowered blood pressure, lowered heart rate and peripheral vasoconstriction) produced by hypothermia, or a direct depression of cell metabolism reducing the energy production necessary to maintain an effective "sodium pump".

All the changes observed were reversed after of the animals were rewarmed.

HYPOTHERMIA, whether induced by surface cooling or by extracorporeal blood-stream cooling or by the use of drugs, now occupies an important place in clinical medicine. This is so particularly in the field of cardiac surgery, and in other surgical operations requiring arrest either of the whole circulation or of the blood supply to a vital organ. Reduction of body temperature enables the body cells, most important of which are those of the central nervous system, apparently to recover normally after their blood supply has been cut off for a much greater length of time than would be possible at normal body temperatures.

Much experimental work has been done during hypothermia, yet surprisingly little investigation has been made of shifts of body water and electrolytes between the body-fluid compartments and the cells. The effects of hypothermia *per se* have been clouded, in many investigations, by various types of anæsthesia, by concurrent surgical intervention and by circulatory arrest.

The present study was undertaken to determine the sodium and potassium exchanges between plasma and skeletal muscle cells, also any alteration in the water concentration in these cells during hypothermia. In order to calculate these changes, it was necessary to determine the serum sodium and potassium contents, the sodium, potassium and water contents of skeletal muscle, and the extracellular space of the skeletal muscle. Changes in the hæmatocrit were also measured.

Cation changes in skeletal muscle in sheep during intravenous anæsthesia have already been investigated and discussed (Hercus and Bowman, 1959). The only variable added throughout the whole of the present series of experiments was the hypothermia; everything else, including the duration of the experiment, was kept constant. Cooling to a body temperature of 28°–30° C. was effected by immersing the anæsthetized, mechanically ventilated animal, in an iced water bath. No surgery was performed; hence no arrest of the circulation or any related procedure was carried out. Thus, the changes solely due to hypothermia could be observed.

METHODS

The eight ewes studied were well acclimatized in their pens, and had been on a constant diet for many weeks. These animals had either been investigated or were later investigated to show the effects of intravenous sodium pentobarbitone anæsthesia on serum and muscle cation. The experiments were done as follows:

Shorn animals, starved for 24 hours, were weighed, and blood was collected for hæmatocrit and serum sodium and potassium estimations; skeletal muscle samples were taken under local anæsthesia for estimation of the muscle sodium, potassium and water

[1] Received on November 5, 1958.
[2] Anæsthetist. This work was made possible by a grant from The Royal Australasian College of Physicians.
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contents, immediately prior to the induction of anæsthesia with sodium pentobarbitone by intravenous injection. The sheep were then intubated and mechanically ventilated with oxygen, the carbon dioxide being removed by soda lime. A second sample of blood was removed after 30 minutes' anæsthesia, for estimation of the hæmatocrit and the serum sodium and potassium contents.

Each animal's bladder was catheterized, and after a rectal or œsophageal thermocouple had been connected to a temperature recorder, the animal was placed in an iced water bath. In the first few experiments rectal temperatures were taken; but later it was recognized

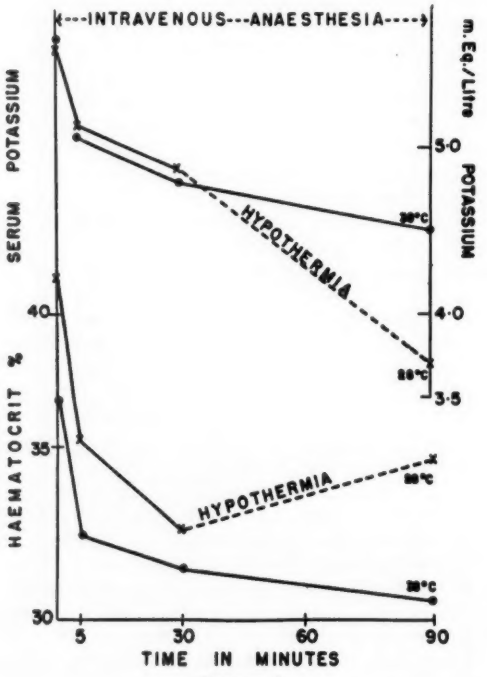

FIGURE I

Serum potassium and hæmatocrit changes in hypothermia and anæsthesia

from other observations (Cohen and Hercus, 1958) that the œsophagus gave a more accurate estimate of the cerebral temperature. Accordingly, in the later experiments, œsophageal temperatures were used as the source of reference for the body temperature.

The usual time required for cooling was 30 to 35 minutes, by which time the temperature was within the range of 31·5°–32° C. When this point was reached, the animal was removed from the bath and placed on the operating table. During cooling signs of shivering were rare, but when they did occur they were readily overcome by injecting a further dose of sodium pentobarbitone. Respirations were controlled throughout by the pump respirator.

On stabilization of the temperature at 28°–29° C. after removal from the bath, three to five microcuries per kilogram of $S^{35}O_4$ were injected intravenously. Blood and urine samples were collected at intervals up to one hour for estimation of $S^{35}O_4$ content. At 30 minutes, extra blood was collected for serum sodium and potassium and hæmatocrit estimations, and specimens of muscle were taken for measurement of $S^{35}O_4$, water, sodium and potassium contents. In some animals extra specimens of blood and muscle were taken at 25 and 40 minutes.

The methods used and the procedure adopted were the same as those used for studying the effects of intravenous anæsthesia (Hercus and Bowman, 1959). Some extra sampling was required in this experiment, because it was thought that hypothermia might cause some delay in the equilibration of $S^{35}O_4$ throughout the body.

RESULTS

Serum Potassium Level

The effect of hypothermia was to cause a considerable fall in the serum potassium level. Intravenous anæsthesia itself lowered this

TABLE I

Serum Sodium, Potassium and Hæmatocrit Changes during Hypothermia (Means and Standard Errors for Eight Animals)

Blood	Before Anæsthesia	During Anæsthesia	Anæsthesia and Hypothermia
Serum potassium level (mEq/l.)	5·6±0·23	4·9±0·14	3·7±0·08
		$p<0.001$	
Serum sodium level (mEq/l.)	145±5·9	141±5·0	144±5·0
Hæmatocrit (percentage)	41·2±1·3	32·5±1·6	35·5±0·6
		$0.001<p<0.01$	

cation in the serum (Figure I), but as a result of the hypothermia it was lowered a further 25%, from 4·9 to 3·7 milliequivalents per litre (Table I).

Serum Sodium Level

No change occurred in the serum sodium level as a consequence of hypothermia (Table I).

Hæmatocrit

After the fall in hæmatocrit induced by anæsthesia, hypothermia caused a significant rise, from 32·5% to 35·5% (Figure I and Table I). However, it is interesting to note that the hæmatocrit still did not return to its pre-anæsthetic level. The fall consequent upon the intravenous administration of the sodium pentobarbitone was always considerable, as is shown in Figure I.

Radioactive Carrier-free Sulphate, $S^{35}O_4$

The rate of equilibration of $S^{35}O_4$ throughout the body in the anæsthetized hypothermic

sheep did not differ from that in the unæsthetized normal animal. Hence the volume of distribution of $S^{35}O_4$, expressed as millilitres per 100 grammes of body weight, in hypothermia is the same as that in the normal animal (Table II).

The "sulphate" space of skeletal muscle, however, showed a significant, indeed remarkable reduction as a result of hypothermia, falling

TABLE II

Effect of Hypothermia on Sulphate Space (Means and Standard Errors for Eight Animals)

$S^{35}O_4$	Before Hypothermia	During Hypothermia
Volume of distribution (ml./100 gm. body weight)	31·6±2·3	31·8±3·0
Muscle space (ml./100 gm. muscle)	14·2±1·6	7·7±0·95
	$p<0.001$	

from 14·2 to 7·7 millilitres per 100 grammes of muscle (Table II). No significant change was found in the "sulphate space" of muscle samples taken at 25, 30 or 40 minutes after injection of the isotope. This confirms the findings stated above—namely, that equilibration of the $S^{35}O_4$ throughout the extracellular fluid of the sheep at 30° C. must have been reasonably complete some 25 minutes after its intravenous injection into the animal.

TABLE III

Changes in Skeletal Muscle Water and Cations in Hypothermia (Means and Standard Errors for Eight Animals)

Muscle	Before Hypothermia	During Hypothermia
Cell water (ml./Kg. muscle fibre)	643±8·8	694±9·6
	$0.001<p<0.01$	
Sodium (mEq./l. fibre water)	27·9±1·5	35·8±1·8
	$0.001<p<0.01$	
Potassium (mEq./l. fibre water)	151·4±2·0	143·0±1·3
	$0.001<p<0.01$	

Muscle Cell Water Content

There was an increase in the amount of intracellular water of 51 millilitres per kilogram. This indicated a shift from the extracellular fluid compartment into the cell. The normal content of 643 millilitres of water per kilogram was elevated to 694 millilitres per kilogram as a result of cooling (Table III).

Muscle Sodium Content

The total amount of intracellular sodium was increased during hypothermia, and its concentration within the cell was increased from 27·9 to 35·8 milliequivalents per litre of fibre water (Table III).

Muscle Potassium Content

The concentration of potassium within the muscle fibre was reduced, but the total amount of potassium was not changed (Table III).

DISCUSSION

It is well known that the normal intracellular-extracellular concentration gradients of sodium and potassium are maintained by the utilization of energy produced within the cell. In the skeletal muscle of sheep, the concentration gradients are as follows:

$$\frac{\text{Na (intracellular)}}{\text{Na (extracellular)}}=\frac{28}{140}\text{ (approximately), i.e., 1 : 5}$$

and

$$\frac{\text{K (intracellular)}}{\text{K (extracellular)}}=\frac{150}{5}\text{ (approximately), i.e., 30 : 1.}$$

These are considerable gradients, and are maintained by the use of a large amount of energy derived from within the cell.

Various conditions, such as anoxia (Hercus *et alii*, 1955) or the presence of metabolic inhibitors (Conway *et alii*, 1954; Fuhrman, 1952; Hercus, 1958) will depress or interfere with the normal cellular metabolism in such a way that significant alterations may occur in these intracellular-extracellular ratios. These facts must be borne constantly in mind, as they are of fundamental importance in understanding and interpreting these results.

Hæmatocrit Changes

The changes in hæmatocrit are shown in Figure I and Table I. The hæmatocrit rises as a result of hypothermia. Intravenous anæsthesia causes a fall in the hæmatocrit, with some hæmodilution, but this is checked and reversed as a result of cooling.

Some previous investigators also have shown a rise in the hæmatocrit during hypothermia (Hegnauer *et alii*, 1950; D'Amato and Hegnauer, 1953; Eliot *et alii*, 1949; Bass and Henschel, 1956). A fall in the plasma volume associated with this rise in the hæmatocrit was observed by Bass and Henschel (1956), and by Swan *et alii* (1953). Similar falls in plasma volume were seen in dogs cooled to 20°–25° C. by D'Amato and Hegnauer (1953). They noted a 12% decrease in plasma volume using T. 1824, but found no change in the plasma proteins or the red-cell volume.

Rodbard *et alii* (1951) found the fall in plasma volume to be as much as 30%. A fall

of 7% in the plasma volume of conscious men, reclining in the nude in a room at 60° F. after spending two hours in a room at 85° to 87° F., was shown to occur by Bass and Axelrod (1956). These changes may all be part of the normal body response to cold described by Lewis (1941), in which there is initially a vasoconstriction of superficial vessels due to the direct action of cold on the skin, followed by reflex transient vasoconstriction and then a central response to the cooled blood causing general vasoconstriction.

The rise in the hæmatocrit with its associated hæmoconcentration may be partly responsible for the increased viscosity of blood occurring during hypothermia, as shown by Hegnauer *et alii* (1950) and by Nichol (1952). All these findings concur with the hæmatocrit changes observed by us during hypothermia in sheep.

However, Smith (1956) found no change in the hæmatocrit in dogs cooled very low, to 14°–25° C., and Swan *et alii* (1953) noted a slight fall in the hæmatocrit during cooling. It is possible that the changes noted by these investigators depended partly on the response to the anæsthetic drugs or other agents used, and thus may not give a true representation of the effects of hypothermia.

Serum Potassium

The changes in serum potassium levels are shown in Figure I and Table I.

Hypothermia in sheep caused a significant fall in the serum potassium level. Similar changes have been observed by other workers; yet the opposite effects have also been recorded. The following brief survey of some of the results of a few well-known investigators will give indications of this complex situation.

A fall in the serum potassium level has been observed by Swan *et alii* (1953), by Covino and Beavers (1957) and by Gollan *et alii* (1957); yet Elliott and Crismon (1947), studying rats, and Smith (1956) and Bigelow *et alii* (1950), studying dogs, found a rise in the serum potassium level. It must be remembered that Bigelow was then using 5% carbon dioxide in oxygen with a low respiratory rate, and consequently his animals may have had quite severe gaseous acidosis.

The serum potassium level was shown by Sealy *et alii* (1954) to vary inversely with the pH of the plasma in animals breathing a mixture rich in carbon dioxide; yet Swan *et alii* (1953) found that the serum potassium level fell in all cases. Sealy *et alii* did not get a straight-line correlation between potassium and carbon dioxide; in fact, the serum potassium level fell in animals with either hypocapnia or hypercapnia. Furthermore, these changes were all within the body, as there was no change in external potassium balance. Swan *et alii* (1953) contended that the pH varied inversely with the carbon dioxide concentration during hypothermia, while Bigelow *et alii* (1954) stated that the pH varied with the rate and depth of respiration. Both these workers and Osborn (1953) showed that intense mechanical respiration could prevent these changes, but the lower the body temperature, the more difficult it became.

The increased solubility of carbon dioxide at low temperatures was pointed out by Rosenhain and Penrod (1951), and by Cranston *et alii* (1955), who also stated that the pH changes were due to this factor. However, Brewin and Neil (1954) and Brewin *et alii* (1956) contended that the acidosis during hypothermia was a non-gaseous acidosis, and that it occurred in animals breathing air or carbon dioxide, and whether they were breathing spontaneously, or on a pump respirator. In view of this, it is possible that the metabolic acidosis occurring in hypothermia may be due to tissue anoxia, and in relation to this, it was shown in 1925 by Koehler *et alii* that anoxia could produce metabolic acidosis. On the other hand, the effect of hypothermia on the body cells may be to interfere directly with normal cell metabolism, possibly depressing it in such a way as to cause acidosis.

This gives some idea of the difficulties of endeavouring to explain a fall in the serum potassium level during hypothermia. It must be remembered that there is very little of the total body potassium content in the plasma, most of it being in the cells. Thus a fall of 50% in the plasma potassium level would be reflected in the cells by a rise of only just over 1%. Such a shift of a cation would be very difficult to trace.

Changes in Muscle Sulphate ($S^{35}O_4$) Space

The sulphate space of skeletal muscle in hypothermia was shown to be reduced considerably (Table II). This appeared to be due to a shift of water and sodium from the extracellular compartment into the muscle fibres.

The rate of equilibration of $S^{35}O_4$ in the hypothermic animal did not differ significantly from that in the normal animal. It appeared that cooling to 30° C. did little to slow down the rate of dilution of the injected $S^{35}O_4$, yet the skeletal muscle sulphate space was greatly lowered.

D'Amato (1954) indicated a decrease in the thiocyanate space and chloride space in dogs

during hypothermia. This reduction in the thiocyanate space was also found by Rodbard *et alii* (1951).

Muscle Fibre Water and Cations

There is a shift of water into the muscle fibre (Table III). This shift of water was also shown by D'Amato (1954) and suggested by Barbour *et alii* (1943). Such a shift was considered to be one of man's natural responses to acute cold exposure by Bass and Henschel (1956).

The "sodium pump" (Dean, 1941) was presumably depressed by the hypothermia, and this enabled extracellular sodium to enter the muscle fibre, at the same time bringing water with it, thus increasing the total sodium content and the sodium concentration in the cell. The concentration of potassium in the muscle fibre was reduced. This was proportional to the amount of water entering the cell from the extracellular fluid. Extra potassium could well accompany this, and yet not be sufficient to raise significantly the intracellular concentration. It is interesting to note that the sodium and potassium ions are working independently of each other—one does not leave the cell as the other enters.

We have already pointed out that lack of oxygen or depression of cell metabolism will result in the inability of the cell to maintain its normal intracellular-extracellular concentration gradients. Hence, as a result of the lowered temperature's reducing cell metabolism and possibly depressing certain enzyme systems, or owing to an impoverished blood supply to the cells caused by peripheral vasoconstriction, or to hæmodynamic changes resulting in a lowered blood pressure, reduced pulse rate and cardiac output, increased viscosity and hæmoconcentration, the efficiency of the "sodium pump" may be impaired, thus allowing sodium and water to enter the cell. Such a response to hypothermia, resulting in depression of intracellular metabolism, could also explain the presence of metabolic acidosis.

CONCLUSION

It is most important to realize that these changes all occur during hypothermia, are completely reversible, and return to normal after rewarming. Knocker (1955) showed changes in liver cells due to hypothermia; then Fisher (1956) produced some very interesting results showing changes in hepatic physiology and in liver slices as a result of hypothermia. One of the most significant features of these findings was the reversible nature of the changes. This work has been criticized because of the fact that the changes were reversible. As clinical results surely indicate a return to normal function of the animal or human being shortly after rewarming, changes of a permanent nature depressing cell metabolism would appear most unlikely to occur. The fact that these changes are reversible must represent one of the most important reasons for the extremely successful clinical applications of hypothermia.

We shall be able to solve these problems, as Bigelow *et alii* (1954) stated, only when we have a "clear understanding of such fundamental problems as oxygen transfer from blood to the tissues and nerve conduction at low body temperature".

ACKNOWLEDGEMENT

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WHIPPLE'S DISEASE

A CLINICO-PATHOLOGICAL STUDY OF THREE CASES[1]

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SUMMARY

The clinical course and autopsy findings in three cases of Whipple's disease are described.

The basic abnormality is considered to be a proliferation of mucoprotein-producing reticulo-endothelial cells, maximally concentrated in the small intestinal lymphatic system, but occurring at other sites including peripheral lymph nodes where, in the earliest stages, these cells can be seen arising in the germ centres. Transitions demonstrable between RNA-containing cells and cells containing mucoprotein are considered to indicate synthesis of the mucoprotein, rather than its phagocytosis by the proliferating reticulo-endothelial cells. Collagenization of lymph nodes and lymphatics is believed to be a result of the activity of these cells and responsible for the observed obstructive phenomena.

The stimulus responsible for the proliferation of the mucoprotein-synthesizing cells is unknown. It may represent a highly differentiated neoplastic process. Again, it may be the outcome of some cellular defect—e.g., enzymatic—whereby there is an abnormal accumulation of a normally synthesized mucoprotein.

Response to steroid therapy in this condition is compatible with the known reticulo-endothelial suppressive action of adrenal cortical steroids. However, this response is not sustained.

THE main features of the disease first reported by Whipple in 1907 are recorded in his original description of a fatal case. His patient was a male medical missionary, aged 36 years, who had suffered gradual loss of weight and strength, steatorrhœa and multiple arthritis. Post-mortem examination revealed deposits of fat and fatty acids in the small intestine, and the mesenteric lymphatics and lymph nodes, many of the latter showing a cystic appearance. Polyserositis was also present. On microscopic examination, Whipple noted fat-containing macrophages, and also large foamy cells which failed to react to fat stains. He considered the basis of the condition to be an obscure defect of fat metabolism.

In 1949, Black-Schaffer first demonstrated that the foamy cells which failed to react to fat stains gave a strongly positive reaction to the periodic acid-Schiff stain, and he concluded that the reacting substance was a glycoprotein. Later, these cells were identified in other sites, particularly in lymph nodes apart from those of the mesentery, in serous membranes where polyserositis occurred, and in vegetations on cardiac valves.

Approximately 60 cases of Whipple's disease have been reported, the majority from America, but some from Great Britain and the Continent. However, the ætiology, pathogenesis and treatment remain to be discovered. Good reviews of the literature are available (Plummer *et alii*, 1950; Hendrix *et alii*, 1950; Peterson and Kampmeier, 1951; Russo, 1952; Puite and Tesluk, 1955).

It is the purpose of this paper briefly to report three further cases of Whipple's disease, and to submit that the primary defect is an abnormal proliferation of mucoprotein-synthesizing cells belonging to the reticulo-endothelial system. Most of the other features of the condition, we believe, result from this proliferation.

CASE REPORTS

CASE I.—An engineer, aged 60 years, presented in December, 1956, with considerable weight loss (approximately 30 kilograms over some years), weakness and anorexia. He had suffered bouts of fever (temperature 100·5° F.), bronchitis, pains in fingers, legs, back and neck, polyuria and lymphadenopathy. There was a neutrophil leucocytosis. Biopsy of an axillary lymph node showed only chronic inflammatory changes.

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[2] Recently Pathologist to the Clinical Research Unit.

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Recently there had been diarrhœa with bulky pale motions. He was dehydrated. The skin showed sallow pigmentation and was atrophic over the fingers, the axillary and inguinal lymph nodes were slightly enlarged and the abdomen was protuberant. The notable laboratory findings were: increased fæcal fat content (up to 55% dry weight), occult blood in the fæces and hypochromic anæmia (hæmoglobin value 7·8 grammes per 100 millilitres). X-ray examination with a barium meal revealed no abnormality, and gastric biopsy showed atrophic gastritis but no foam cells.

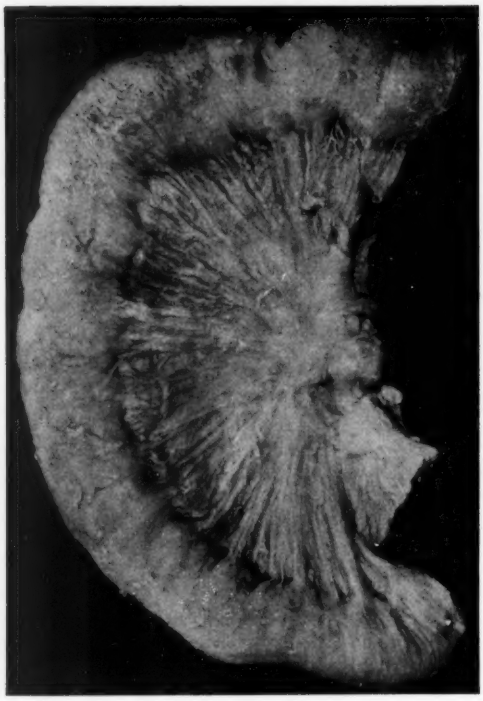

FIGURE I

Case I. Male, aged 61 years, who died of Whipple's disease and bronchopneumonia. The loop of ileum with its mesentery shows serosal thickening, fibrous tags and prominent radiating lymphatics. A lymph node is visible in the middle of the cut edge of the mesentery

Laparotomy was undertaken to investigate the possibility of a neoplasm, and this revealed firm, white mesenteric lymph nodes enlarged up to two centimetres in diameter, which on microscopic examination showed the characteristic features of Whipple's disease. There was also roughening of the serosa of the small intestine, liver and spleen. Cortisone (75 milligrammes per day given orally), blood transfusion and a diet of low fat content were of minor benefit. He died three months later from bronchopneumonia.

At autopsy, the body weighed 43 kilograms. Ecchymoses were present on the arms. Examination of the body cavities revealed polyserositis; the peritoneal cavity was as described at operation, the pleural surfaces were roughened by fine fibrous tags and the pericardial cavity was obliterated. The lymphatics of the mesentery (Figure I) and lesser omentum were prominent. The thoracic duct was thickened, but not obstructed. The appearance of the mesenteric and para-aortic lymph nodes was similar to that observed at laparotomy. The axillary and inguinal nodes were slightly enlarged; the cervical lymph nodes appeared normal. The mucosa of the small intestine was thickened and pale with a granular surface. Hard white vegetations were present on the aortic valves, with recent thrombus on the anterior cusp (Figure II). Mild nephrosclerosis and adrenal cortical atrophy were present. In the lungs emphysema and bronchopneumonia were found.

The microscopic findings were as follows. The lamina propria of the small intestine was crowded with the classical pale foamy cells, 20 to 30μ in diameter, with small, dark, eccentric nuclei (Figure III). Plasma cells were numerous, some with Russell bodies, others

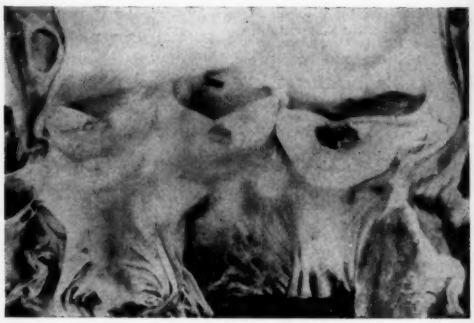

FIGURE II

Case I. Aortic valves with hard calcified vegetations on the cusps, recent thrombus on the anterior cusp

with typical plasma-cell nuclei and cytoplasmic vacuolation similar to that seen in the larger foamy cells. With Unna-Pappenheim staining (Figure IV), transitions could be seen from cells with wholly pyroninophil cytoplasm to pyroninophil cells with patchy vacuoles to the large foamy cells, the vacuoles of which were separated by strands of pyroninophil material. Periodic acid-Schiff staining (Figure V) likewise showed a range of reaction from faintly staining plasma cells to more deeply staining larger cells with plasma cell nuclei, to the classical cells with intensely staining cytoplasm. The lymphatics in the submucosa and serosa were dilated. In the mesenteric lymph nodes the sinuses were dilated and contained fat (Figure VI). Scattered foam cells were present, together with multinuclear fat-containing macrophages. Rounded areas of hyalinization were fairly numerous. The lymphatics of the mesentery showed thickened walls with partial obstruction of the lumen (Figure VII). Small numbers of foam cells were present in the posterior mediastinal, but not in axillary or inguinal nodes. A few such cells were present in the aortic valve vegetations.

CASE II.—In 1939 a man, aged 44 years, began to suffer from bouts of diarrhœa and vomiting. These continued with progressive weight loss (from 70 to 51 kilograms) until his death in 1947. Additional features of his illness were the development of a

chronic cough in 1943, with fever and night sweats (repeated searching failed to find acid-fast bacilli), and an acute urinary infection followed by epididymo-orchitis in 1946. In the later stages there were hypotension (blood pressure 80/40 mm. Hg) and hypochromic anæmia (hæmoglobin value 8·1 grammes per 100 millilitres).

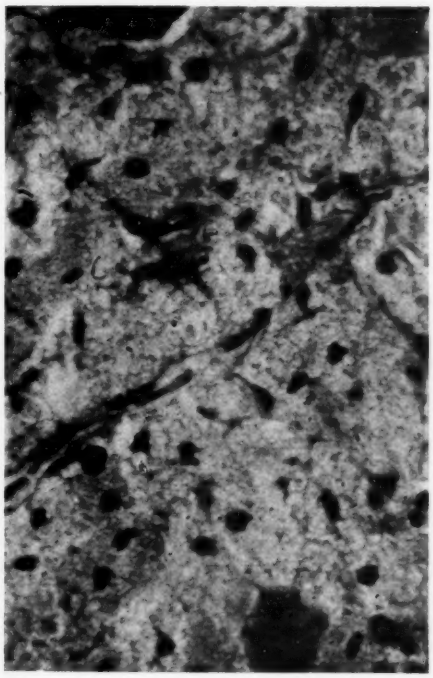

FIGURE III

Case I. Small intestinal mucosa showing lamina propria packed with cells 20μ to 30μ in diameter, with small round nuclei frequently eccentric in position, and pale-staining foamy cytoplasm. (Hæmatoxylin and eosin, ×400.)

At autopsy, features of polyserositis were present—fibrin coating of the small intestine, obliteration of the pleural cavities, and dense fibrous adhesions across the pericardial cavity. The mesenteric lymph nodes were enlarged up to five centimetres in diameter. The appearance of the cut surface varied, some being fibrotic, others showing yellow streaking and some having liquid contents. The axillary, inguinal and intrathoracic lymph nodes appeared normal. The mucosa of the small intestine was blackened and the large intestine was pigmented to a lesser degree.

The microscopic findings were as follows. The lamina propria of the small intestine was crowded with cells of similar appearance to those in Case I. In the submucosa were old hæmorrhages and hæmosiderin-containing macrophages (Figure VIII). So far as we are aware, this finding has not previously been reported in Whipple's disease. The lymphatics of the serosa and of the mesentery were dilated and contained fat. Examination of mesenteric lymph nodes showed dilated fat-containing sinuses, collections of foam cells and numerous hyaline areas replacing follicles.

CASE III.—A labourer, aged 49 years, gave a history of three months' duration of lassitude, loss of appetite and diarrhœa. He was severely ill and dehydrated. The fæcal fat content was increased (44% dry weight), occult blood was present in the fæces, the glucose tolerance test produced a "flat" curve, and hypochromic anæmia was present. X-ray examination with a barium meal suggested external pressure on the first part of the duodenum. A diet of high protein content and blood transfusion were given with some improvement, but no benefit was derived from either folic acid or a gluten-free diet. He returned to work, but was readmitted to hospital one year after the onset of his symptoms with continued loss of strength and weight. Small lymph nodes were now palpable in the posterior cervical triangle and glossitis was present. His blood pressure was 95/50 mm. Hg. The terminal stages were characterized by continuing diarrhœa and progressive emaciation.

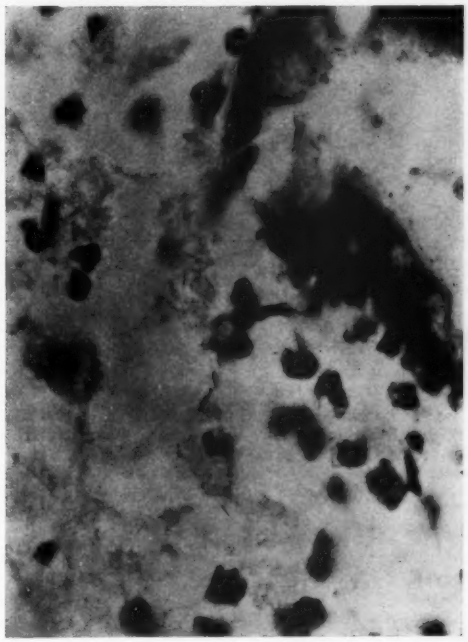

FIGURE IV

Case I. Lamina propria of small intestine with portion of crypt of Lieberkühn at top right. A plasma cell with pyroninophil cytoplasm is present towards the lower right part of the figure. There is a pyroninophil cell showing patchy cytoplasmic vacuolation about the middle of the left side of the figure and to the right of this a foamy cell with pyroninophil cytoplasmic strands. (Unna-Pappenheim, ×1200.)

At autopsy, the body weighed 48 kilograms. The height was 150 centimetres. The outstanding findings were enlarged abdominal and cervical lymph nodes, obliterative pericarditis, and cardiac valvulitis with warty vegetations on the cusps of all valves except the

aortic. The mesenteric and para-aortic lymph nodes measured up to four centimetres in diameter. The cut surface of many presented the striking appearance of honeycombing by minute cystic spaces recorded in most other cases of Whipple's disease, but not so notable in Cases I and II. In the enlarged cervical nodes this

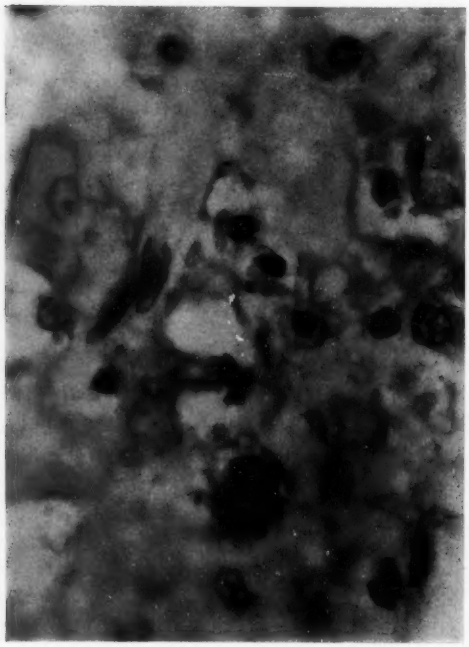

FIGURE V

Case I. Lamina propria of small intestine. A plasma cell which stained faintly with PAS is present above the centre. Larger cells with plasma-cell nuclei, which stained more strongly with PAS, are present near the top right edge and below the centre. (Periodic acid-Schiff, ×1200.)

appearance was less conspicuous. Enlarged nodes about the head of the pancreas were responsible for the duodenal deformity.

The findings on microscopic examination were as follows. In the small intestine and mesenteric lymph nodes the appearances were essentially the same as in the previous cases, apart from the much greater dilatation of lymph node sinuses (Figure IX). There were collections of the characteristic foamy cells in the cardiac vegetations. The presence of the classical features of Whipple's disease in cervical lymph nodes (Figure X) was of great clinical importance. The sinuses and efferent lymphatics were dilated and contained fat. The cortex, and to a lesser extent the medullary cords and sinuses, contained collections of pale foamy cells. In the cortex these were arising in germ centres (Figure XI). The amount of reticulin was increased at these sites, and in some follicles it had progressed to collagen formation; this suggested an earlier stage in the development of the extensive hyaline areas seen in the mesenteric nodes in Cases I and II.

DISCUSSION

Numerous theories as to the ætiology of Whipple's disease have been put forward. Many writers consider that a defect of the intestinal mucosa is the primary defect in the disease (Whipple, 1907; Black-Schaffer, 1949; Upton, 1952; Christie and Galton, 1952; Puite and Tesluk, 1955). Others favour a mechanical obstructive basis (Jarcho, 1936; Hill, 1937; Clemmesen, 1945; Rosen and Rosen, 1947; Newman and Pope, 1948; Chapnick, 1948). No thoracic duct obstruction has ever been demonstrated in Whipple's disease (Whipple, 1907; Sailer and McGann, 1942; Upton, 1952). However, Crane and Aguilar (1957) demonstrated chronic proliferative lymphangitis and obliteration of efferent lymphatics at the base of the mesentery of the small intestine. Because of such features

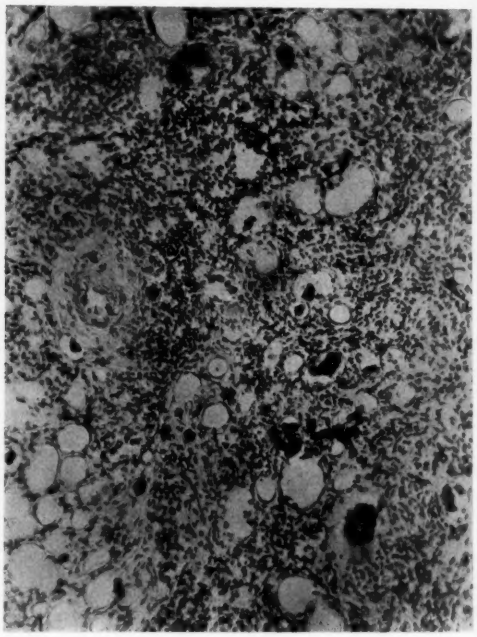

FIGURE VI

Case I. Mesenteric lymph node, showing dilated lymph sinuses, some containing extracellular fat globules. (Scharlach R, ×100.)

as fever, arthralgia, endocarditis, polyserositis and response to steroids, Jarcho (1936), Clemmesen (1945), Plummer *et alii* (1950), Peterson and Kampmeier (1951) and Crane and Aguilar (1957) identify this condition with the rheumatic or "collagen" diseases; Jarcho, Clemmeson

and Crane and Aguilar consider that a process of this nature is the basis of the mesenteric lymphatic obstruction.

The theory of an intestinal defect in the handling of fat as the basic abnormality is difficult to accept, in view of the finding in Case III of cervical lymph nodes with dilated fat-containing sinuses and lymphatics similar in appearance to the mesenteric lymph nodes. In this situation the fat concerned is fat being mobilized from the tissues, the lymphatic system acting as a vehicle of transfer back to the blood-stream for those lipids not used by the cells (Yoffey and Courtice, 1956). It would seem that the explanation of these appearances must be either obstruction to onward flow of lymph at some point, or some alteration in the

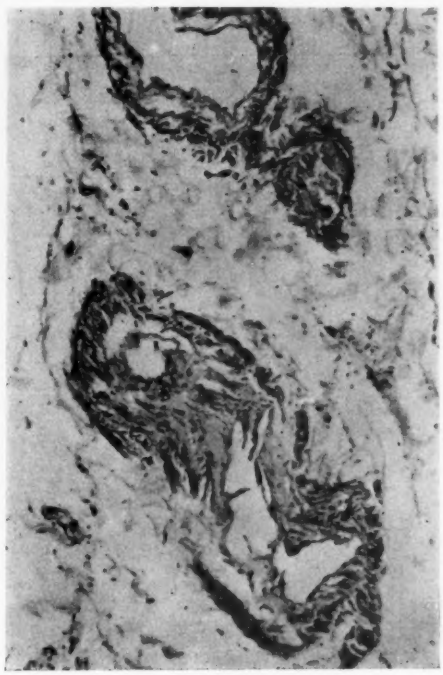

FIGURE VII

Case I. Mesenteric lymphatics, showing thickened walls. Glycoprotein-containing cells are also present in the interstitial tissue. (Hæmatoxylin and eosin, ×40.)

fat as a result of its contact with lymph node tissues.

Mechanical lymphatic obstruction clearly seems a reasonable explanation for the appearances in the lymph nodes and lymphatics in these patients. It was shown in the mesenteric lymphatics in Case I of our series, and was demonstrated by Crane and Aguilar. However, these authors consider that the proliferation of the intestinal mucoprotein-containing cells represents merely a phagocytic response to

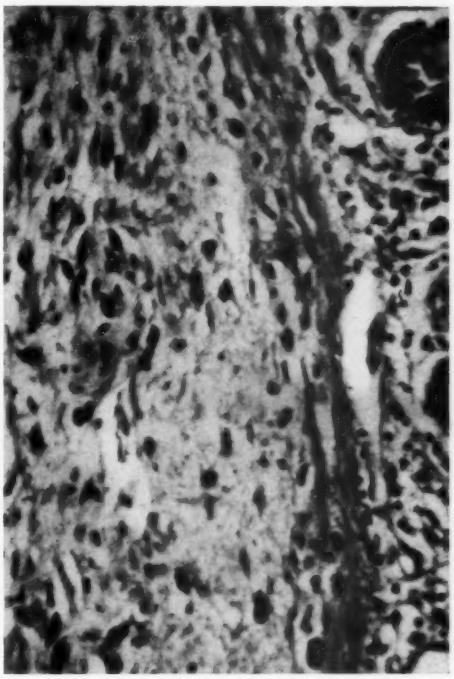

FIGURE VIII

Case II. A man, aged 52 years, dead of Whipple's disease; small intestinal submucosa with macrophages containing iron pigment. (Prussian blue, ×300.)

absorbed intestinal contents whose further progress is prevented by the lymphatic obstruction.

As we stated at the outset, we believe the proliferation of the mucoprotein-containing foamy cell to be the fundamental defect in Whipple's disease. We submit that the thickening and fibrosis of the walls of lymphatics are a result of this proliferation, and that the striking picture of dilated sinuses in the lymph nodes results from the obstruction thus produced.

Moreover, we consider that the evidence available points to the mucoprotein's being synthesized by the cells rather than to its being absorbed by them. Histochemical studies of PAS-staining cells of reticulo-endothelial origin have been carried out by Pearse (1949), White (1954) and Teilum (1956). Pearse, in a study of

the cytochemistry of Russell bodies in plasma cells, presented evidence that these contained mucoprotein. He also demonstrated the presence of mucoprotein in some normal plasma cells, and observed intermediate stages between plasma cells with faintly PAS-staining cytoplasm

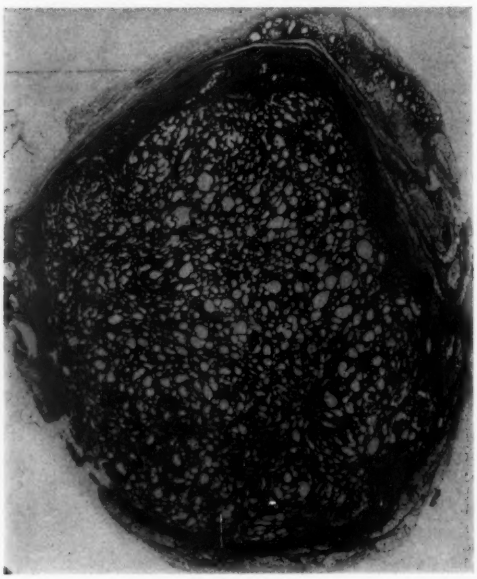

FIGURE IX

Case III. A man, aged 49 years, dead of Whipple's disease, showing the honeycombed structure of the mesenteric lymph node, with dilatation of both afferent and efferent lymph sinuses. (Hæmatoxylin and eosin, ×4·5.)

and those that stained strongly; there was an inverse relationship between the RNA content of the cytoplasm as indicated by pyroninophilia, and its mucoprotein content as indicated by PAS staining. He considered that this indicated secretion rather than absorption of the mucoprotein.

Not only in plasma cells forming Russell bodies has an inverse relationship between the RNA and the mucoprotein content of the cytoplasm been demonstrated; Teilum (1956) noted the same occurrence in reticulo-endothelial cells proliferating in response to a variety of stimuli. He considered that PAS-staining cells, pyroninophil cells and intermediate cell types (containing both PAS-staining and pyroninophil material in the cytoplasm) were characteristic functional stages of various types of reticulo-endothelial cell. Also, he considered that these cytoplasmic changes were directly involved in the genesis of a variety of lesions of mesenchymal tissue, including the formation of hyaline.

This inverse relationship between RNA and mucoprotein cytoplasmic content was observed in Case I, in which Unna-Pappenheim staining showed transitions from pyroninophilic cells to cells with small vacuoles to the large foamy cell, which consisted of clear mucoprotein separated by fine strands of pyroninophil material. With PAS staining a similar range was seen, from faintly staining plasma cells, to more deeply staining cells still with recognizable plasma cell nuclei, and thence to cells having an intensely PAS-staining cytoplasm. Table I shows the histochemical similarities between the mucoprotein-containing cells of Whipple's disease, the reticulo-endothelial cells described by Teilum, and the Russell bodies studied by Pearse.

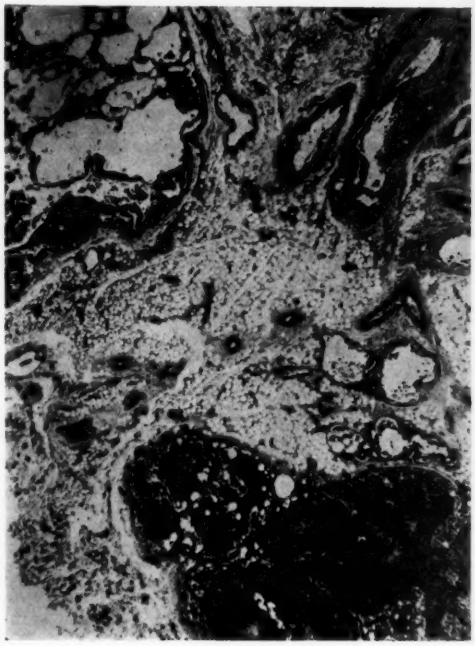

FIGURE X

Case III. Adjacent cervical lymph nodes, one showing only slight, the other marked dilatation of sinuses. Afferent and efferent lymphatics are dilated. (Hæmatoxylin and eosin, ×7.)

At the site where the minimal lesion in our patients was seen—namely, the cervical lymph nodes in Case III—it is noteworthy that even small collections of mucoprotein-containing cells were associated with increased reticulin and

collagen formation. At some sites in the node there were hyaline areas of considerable size, and in numerous mesenteric nodes extensive hyalinization was a striking feature. We consider that the thickening and fibrosis seen in the walls of the mesenteric lymphatics in Case I and suspected in the cervical lymphatics in Case III can be attributed to the proliferating mucoprotein cells. Collections of these cells were not infrequently noted within lymphatics. Similarly, in most instances these cells were to be seen in the lesions of polyserositis and in the cardiac vegetations.

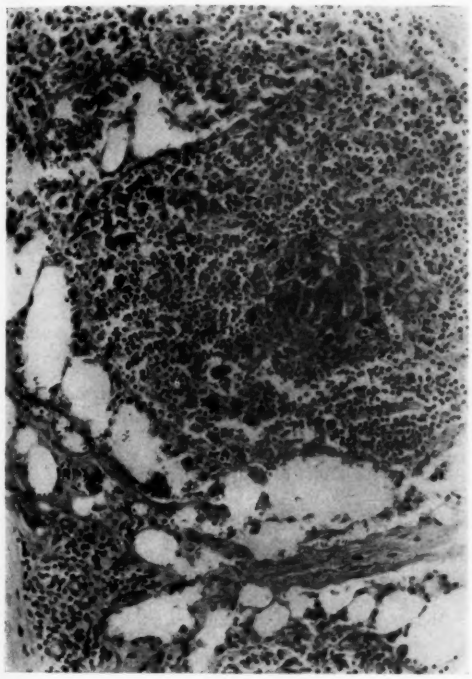

FIGURE XI

Case III. Cervical lymph node, showing nest of macrophages in the centre of the follicle, many of them containing granules of glycoprotein. (Periodic acid-Schiff, ×125.)

The stimulus to the cellular proliferation in Whipple's disease is not apparent. In animal experimental work on mucoprotein production (White, 1954; Teilum, 1956), bacteria and their toxins were the agents responsible. Case reports of Whipple's disease provide no evidence of any such process. The massive and widespread proliferation of the mucoprotein-synthesizing cells suggests the possibility of neoplastic proliferation of the nature of a reticulosis, though the absence of destructive infiltration of tissues and the functionally mature character of the cells are contrary to the traditionally accepted criteria of malignant neoplasia.

Some support for a genetic basis to the condition is given by reports of suggestive family histories and the occurrence of the disease in siblings (Puite and Tesluk, 1955; Scott and Hosie, 1957). One might postulate an enzyme defect as responsible for the continued overproduction of mucoprotein.

In a disease whose natural history is characterized by remarkable fluctuations, assessment of any therapy is difficult. However, remissions have been induced with cortisone (Jones *et alii*, 1953; Lepore, 1954; Wang *et alii*, 1956) and with ACTH (Plummer *et alii*, 1953; Puite and Tesluk, 1955). The mode of action may be related to their known reticulo-endothelial suppressive activity; but such remissions, particularly with regard to malabsorption, can be only minimal when obstruction of lymphatics has become extensive (Crane and Aguilar, 1957).

TABLE I

Histochemical Reactions of the Cytoplasm of the Mononuclear Cells in Whipple's Disease.—Comparison of Combined Results in Cases I, II and III, with Results of Other Authors, and with the Staining Reactions of the Glycoproteins in Russell Bodies and Reticulo-Endothelial Cells

Stain or Test	Results	Other Authors[1]	Glycoprotein in Russell Bodies and R.E. Cells[2]
Hæmatoxylin and eosin	Pale or eosinophilic refractile granules, with basophilic strands or diffuse eosinophilia	Variable cytoplasmic eosinophilia or basophilia	Eosinophilic refractile granules, with basophilic strands
Periodic acid-Schiff	Intensely positive	Intensely positive	Intensely positive
Mucicarmine	Weakly positive	Weakly positive	——
Glycogen	Negative	Negative	Negative
Acid mucopolysaccharides	Negative	Variable	Negative
Metachromasia	Negative	Variable	Negative
Methyl green-pyronin	Pyroninophilia mainly in small mononuclear cells. Large cells weakly pyroninophilic	——	Pyroninophilia around globules
Water solubility	Negative	Negative	Negative

[1] Black-Schaffer, 1949; Christie and Galton, 1952; Upton, 1952; Jones *et alii*, 1953; Casselman *et alii*, 1954.
[2] Pearse, 1949; White, 1954; Teilum, 1956.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Sir Macfarlane Burnet and Dr. Ian Wood for their interest and for their assistance in the preparation of this paper. Permission to publish the details of Case II has been given by the Chairman of the Repatriation Commission, Melbourne. Permission to report Case III has been given by Professor C. V. Harrison and Professor Russell Fraser, of the Postgraduate Medical School, London, in whose departments the patient was examined by one of us (M.R.). We are grateful to Dr. A. R. Parkin, Miss E. Earle, Mr. R. Inglis and Mr. E. A. Matthaei for their assistance in the preparation of the sections and photographs.

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PULMONARY ALVEOLAR PROTEINOSIS[1]

REPORT OF A CASE

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From Green Lane Hospital, Auckland, New Zealand

SUMMARY

A case of pulmonary alveolar proteinosis is reported, in the belief that this is the first case to be recognized in New Zealand and possibly in Australasia. This condition, of unknown ætiology, was first described in June, 1958, by Rosen, Castleman and Liebow. This chronic and sometimes fatal illness often begins with a pneumonic episode followed by dyspnœa, cough, chest pain and loss of weight. Characteristically, the symptoms, physical and radiological signs and pathological changes are limited to the lower part of the respiratory tract and the lung parenchyma. Equally characteristically, the physical signs fail to reflect the often gross radiological changes of extensive, pulmonary infiltration. The essential histological feature is the plugging of alveolar and other distal air spaces in the lungs with a proteinaceous material rich in lipids. This patient was ill for approximately four years before making a complete recovery.

UNDER the title of "Pulmonary Alveolar Proteinosis", Rosen, Castleman and Liebow (1958) record 27 cases of an obscure pulmonary disorder with distinctive features not previously described. The distinguishing characteristics of this chronic and sometimes fatal condition rest on the radiographic appearances and on the histological picture in lung biopsy or autopsy material. At the height of the illness, the lung-fields are obscured by a more or less symmetrical discrete or confluent mottling, involving more particularly the perihilar and basal zones. The mottling is variously described as "fluffy" or "feathery", and mimics the "butterfly" distribution of the abnormal shadowing seen in acute pulmonary œdema. The essential histological feature is the filling of alveolar and distal air spaces with a proteinaceous material which stains with P.A.S.

The clinical picture is not as precise as the radiographic and histological findings, but there are a number of distinctive features common to the cases originally reported and to the subject of this communication. Most of the patients have been adults between 20 and 50 years of age. The exact time of onset of the disease has been difficult to establish, but in a majority of the cases a pneumonic or pneumonia-like illness figured in the introductory history. The dominant symptoms were steadily increasing dyspnœa and productive cough, and some patients complained of lassitude, chest pain and loss of weight. Fever was usually absent.

The physical signs were quite out of keeping with the often extensive and gross radiographic changes. In the original group of patients, râles were heard in only four cases and resonance was impaired in only one. Finger-clubbing was seen in three of the original cases, and of these three cases two were fatal. Cyanosis was recorded as occurring terminally in five cases.

HISTOLOGICAL FEATURES OF THE ORIGINAL CASES

The original article provides an exhaustive study of the histological features and of the chemical and physical properties of the so-called "proteinaceous" material. We content ourselves with extracts as a basis for comparison with the histological findings in our own case.

The most characteristic feature was the plugging of alveolar spaces and terminal bronchioles with a granular, acidophilic and so-called "proteinaceous" material. This material was not demonstrated in the interstitial tissues. A few, or occasionally many, erythrocytes were encountered within this

[1] Received on November 11, 1958.
[2] Physician-in-charge, Chest Department.
[3] Director, Pathology Department.
[4] Senior full-time Physician, Chest Department.

substance, but hæmosiderosis was not a feature. The material stained strongly with P.A.S. All stains designed to aid in the demonstration of microorganisms gave negative results. The authors considered that much of the proteinaceous material was formed by a transformation of the so-called "septal cells" of the alveolar walls. An increase in the number of septal cells is regarded as representing an early stage in the development of the characteristic lesion. As these cells multiply in number and size, their cytoplasm becomes transformed by the appearance of P.A.S.-staining granules and floccules. Finally, these cells slough into the lumen of the alveolus; the nuclei degenerate and the cytoplasm disintegrates into the proteinaceous material of the fully developed plug. The authors stress the absence of cellular infiltration in the interalveolar septa and the rarity of plasma cells, in contrast to their usual abundance in pneumocystis infections, which has some features in common with the condition under consideration. In some specimens lymphocytic infiltrations were seen in the wall of bronchioles, but seldom extending into interalveolar septa. Small vessels generally did not appear to be congested, and the lymphatics were not prominent.

CASE REPORT

The patient, a Chinese male, aged 47 years, first came under the care of one of us (C.McD.) on October 17, 1952. He was a fruiterer and greengrocer. Born in Canton, he had apparently come to New Zealand for the first time in 1914. There was a certain language difficulty, and his earlier history was untangled and in part documented and assembled in piecemeal fashion. In the interest of clarity the history is given as finally assembled with some stream of continuity.

For some reason which remains obscure, his chest was radiologically examined in Auckland for the first time on November 3, 1949. This film (Figure I) was frankly abnormal; but apparently no further action was taken at the time, and shortly afterwards the patient moved first to Wellington, and later, somewhat surprisingly for a Chinese long-domiciled in New Zealand, to England. While in London he was referred, and later admitted, to Brompton Hospital on August 28, 1950. We are indebted to the Medical Registrar for a detailed summary of his case notes while in hospital, and the following relevant information is taken from this précis.

On his admission to Brompton Hospital, he gave a history of increasing shortness of breath over the preceding 12 months, with cough and expectoration of mucoid sputum. He said that he had seen some blood in his sputum 12 months previously. The earlier history included an operation for appendicitis at the age of 20 years, and pneumonia in 1947. It is noted that his history tended to vary somewhat on different occasions. On examination, he was found to have slight cyanosis of the lips, but was not breathless at rest. Gross finger-clubbing was recorded. Mobility of the chest was limited, and moist sounds were present at both lung bases. The erythrocyte sedimentation rate was recorded as six millimetres in one hour (? Westergren), and the blood pressure as 128/92 mm. Hg. There was no evidence of congestive heart failure, and no palpable enlargement of liver, spleen or superficial lymph nodes. The vital capacity was recorded as 2200 millilitres. There was no anæmia or leucocytosis. The Wassermann and Kahn tests gave negative results. The chest X-ray film was reported as showing diminished translucency of both lung bases, with very fine nodular shadows throughout. Tomography failed to reveal any cavitation or enlargement of hilar lymph nodes. The question of a possible hæmosiderosis was raised by the radiologist. Repeated sputum examinations by concentration and culture failed to reveal acid-fast bacilli. A culture on Sabouraud's medium yielded a scanty growth of a yeast considered to be non-pathogenic. Nevertheless, the possibility of a fungous infection was canvassed, and the patient was discharged from hospital on September 13, 1950, taking a small dose of potassium iodide, and instructed to return for follow-up investigation. He did not continue treatment, and did not report for further supervision.

Not long after his discharge he set out on his return to New Zealand by way of Australia where, on April 7, 1951, he was admitted to a Sydney hospital with suspected infectious hepatitis. However, on April 16 a cholecystostomy or cholecystotomy was performed and he was discharged from hospital on May 8. One brief report from this hospital makes no mention of gall-stones having been removed.

The history then moves on to September 14, 1952, after his return to New Zealand, when he was admitted to the Auckland Public Hospital for investigation of acute upper abdominal pain. On the day after his admission the pain had relented, but he was feverish, sweating and dyspnœic, and was found to have impairment of resonance over both lung bases, with basal moist sounds more marked on the left side than on the right. A provisional diagnosis of bilateral basal bronchopneumonia was made, and he was given both penicillin and sulphadiazine. The fever promptly settled, and he was discharged from hospital as recovered on October 3, although an X-ray film of his chest taken on September 29 was reported as showing little change from that of November 3, 1949.

He first came under our care on October 17, 1952. There was nothing significant to add to the history of increasing shortness of breath, cough and some expectoration over a period by this time of some three years. The only significant signs on physical examination were frequent, shallow respirations, gross finger-clubbing (but not of the "drum-stick" type) and impairment of resonance over the lung bases, with enfeebled breath-sounds and showers of fine crepitations. Some small, firm lymph nodes were palpable in the right groin. Although he said that he had lost one stone in weight in the previous four weeks, he was still well covered. None the less, he appeared to be a very sick man. At this time no precise information was available regarding earlier investigations, and a tentative diagnosis of bizarre pulmonary tuberculosis was made. He was given a three weeks' course of streptomycin injections and isoniazid while he was making arrangements for the carrying on of his business and pending his admission to hospital. There was no response to treatment. His weight fell, his dyspnœa increased, and the abnormal shadowing in his lung fields extended and intensified, as is shown in the film of November 7 (Figure II).

He was admitted to Green Lane Hospital on November 11, 1952. His hæmoglobin value was estimated at 15·6 grammes per 100 millilitre; the

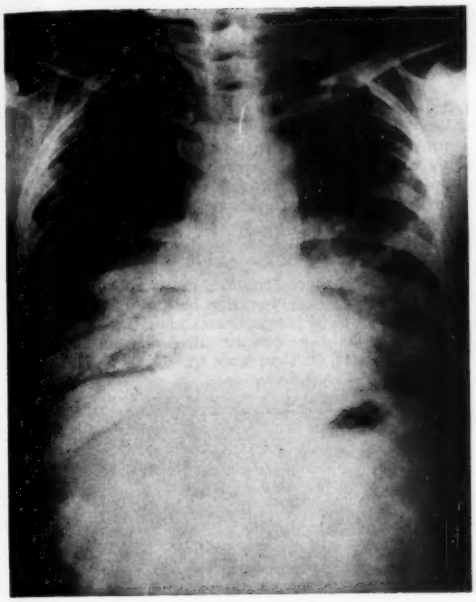

FIGURE I
X-ray film of chest, November 3, 1949

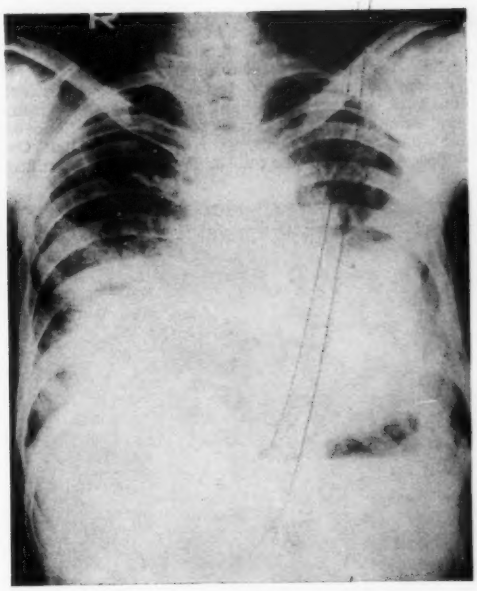

FIGURE II
X-ray film of chest, November 7, 1952, showing a moderate increase in the extent of the parenchymal infiltration over a period of three years

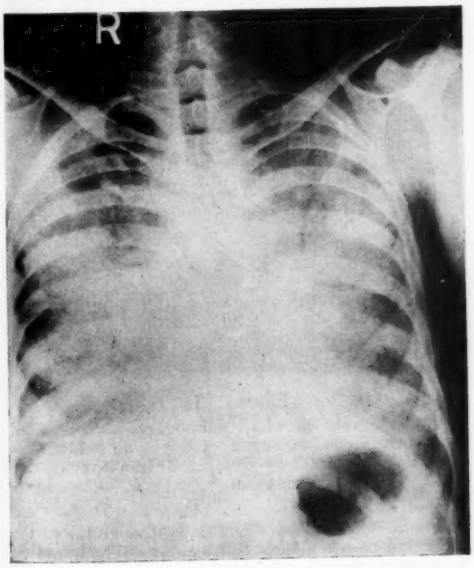

FIGURE III
X-ray film of chest of December 10, 1952, showing almost complete involvement of lung fields at the height of the illness

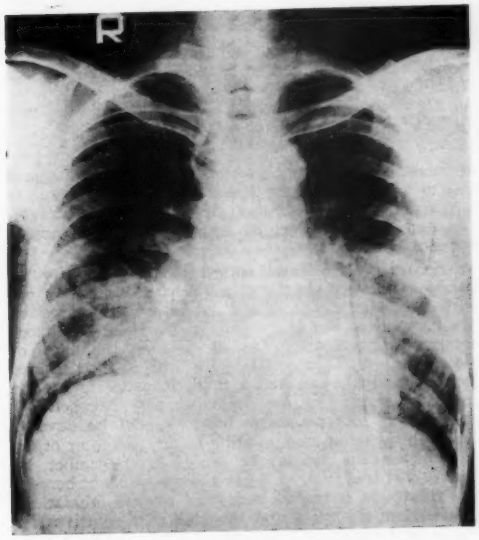

FIGURE IV
X-ray film of chest of May 5, 1953, illustrating a stage in the process of recovery

leucocytes numbered 6900 per cubic millimetre; the erythrocyte sedimentation rate was three millimetres in one hour (Westergren). Empirical liver function tests gave normal results. The results of the Wassermann and Kahn tests remained negative. Numerous specimens of light mucopurulent sputum were examined for acid-fast bacilli, neoplastic cells and significant pathogens, with negative results. On two occasions yeast-like cells or Monilia were identified

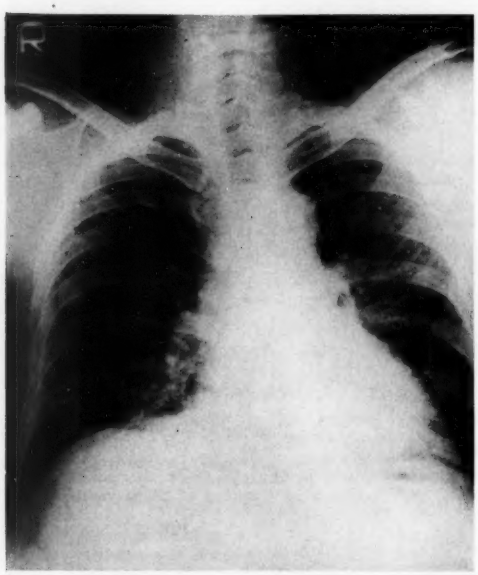

FIGURE V

X-ray film of chest of June 28, 1954, showing complete clearing of parenchymal infiltration. There is some minor residual exaggeration of medial basal lung markings on the right side

in sputum, and from one specimen *Candida krusei* was cultured. A biopsy specimen of a right inguinal lymph node was taken on December 15, but was reported as showing no more than a reactive hyperplasia.

The patient's general course in hospital may be summed up very simply as a relentless deterioration, with increasing dyspnœa and cyanosis and progressive involvement of the lung fields to the point of almost complete opacification, as shown on the film of December 10 (Figure III). He lost a further two stone in weight, and had a mild, irregular fever, the temperature ranging for the most part between 99° and 100° F. At no stage, however, did he develop any signs or symptoms relating to any tissue or organ other than the lungs. In November-December he had a short trial of deep X-ray therapy without benefit. His treatment consisted mainly of oxygen inhalations for relief of dyspnœa and cyanosis and sedatives for cough and insomnia. He was also given potassium iodide on the slender evidence of a possible monilial infection. As there was no specific indication for the exhibition of penicillin or broad-spectrum antibiotics they were not used, except for a very short course of penicillin injections to cover a limited right thoracotomy and lung biopsy on February 8, 1953. Details of the histological findings are given below.

From about this time the patient began to show some sign of improvement and within a matter of a few weeks appeared to be well on the road to recovery. Even as early as February 16 there had been appreciable radiological clearing, and by May 5 the X-ray appearances were comparable with those of November 3, 1949 (Figure IV). Further improvement followed, and by June 28, 1954, the lung fields were clear except for some local increase in lung markings in the right cardio-phrenic angle (Figure V). He was last examined on March 11, 1958, and has remained well since except for some trouble with his gall-bladder.

It is unfortunate that circumstances make it impossible to decide whether recovery in this case was spontaneous or in response to treatment. On the grounds that he was possibly suffering from a monilial infection, he was, on December 29, 1952, given a simple

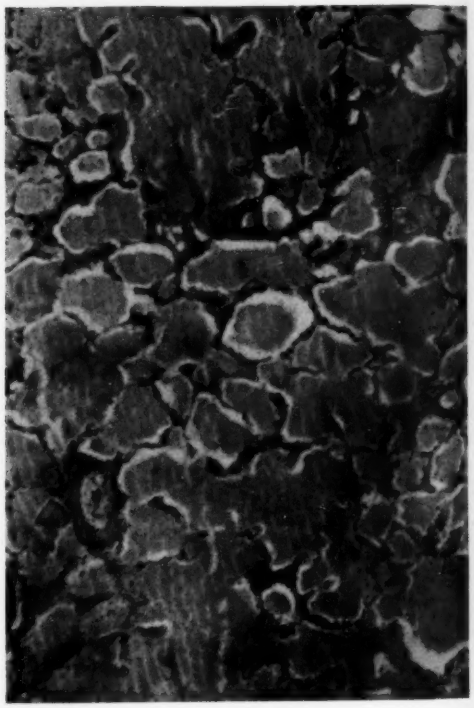

FIGURE VI

Photomicrograph showing pulmonary alveoli filled with proteinaceous material. There is no evidence of interstitial inflammatory change. (Hæmatoxylin and eosin stain, ×70.)

potassium iodide mixture containing five grains to the drachm, to be taken three times a day. The intention was to increase the dose at weekly intervals to two and finally three drachms. In error he was given ounce instead of drachm doses of this mixture for some weeks in January, 1953. Over this period he was having at least 120 grains of potassium iodide daily, and possibly double or treble this amount. The error was discovered

on February 3, 1953, and the dose reduced to 45 grains daily. It is considered possible that the extravagant intake of iodide may have played some part in this patient's recovery.

Histological Findings

The original histological report (S.E.W.) was as follows:

One specimen consists of a small wedge of dark, red, lung tissue measuring 3 × 1·5 × 1 cm. It is rather firm and contains scattered pale nodules.

The Pleura. This is of normal thickness and structure. Interlobular septa are also normal.

The Alveoli. Almost the entire alveolar system is filled with a peculiar, finely granular, eosinophilic, inspissated material which is broken up by the microtome knife into a series of slabs reminiscent of the properties of thyroid colloid material. In many areas this material includes large numbers of red cells in various stages of disintegration and in some sites the alveoli are filled with blood. Pigment granules are present in very small numbers only. Some very large pale histiocytes are seen in many alveoli and in some peripheral areas they fill the alveoli. They do not contain pigment granules although a second type of smaller histiocytes filled with granules—the typical "heart-failure" cells—are found lining the alveoli and in the interstitial tissues of the hæmorrhagic areas. No air-containing alveoli are seen in any of the blocks examined.

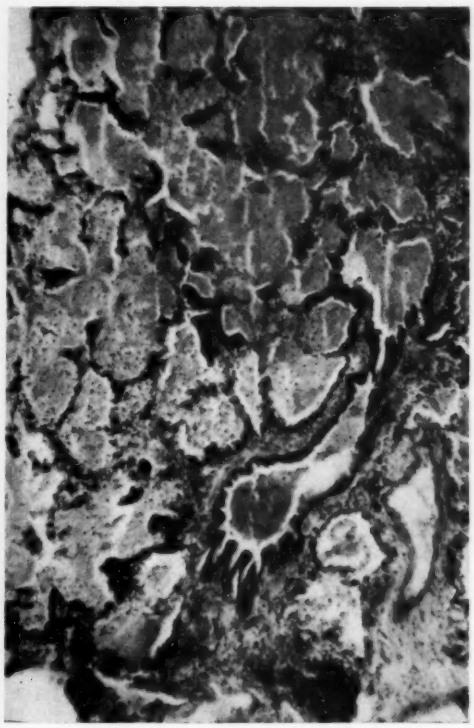

FIGURE VII

Photomicrograph showing proteinaceous material within a bronchiole. (Hæmatoxylin and eosin stain, ×70.)

The Alveolar Septa. The anatomical distribution of alveoli is normal. Many septa are of normal thickness while others show a definite fine fibrous thickening. There is a general diminution in the number of capillaries visible in the septa. The septal cells are increased in number and in some cases form a continuous lining to alveoli. Occasional neutrophil and eosinophil leucocytes are found in the septa but there is no indication of inflammation.

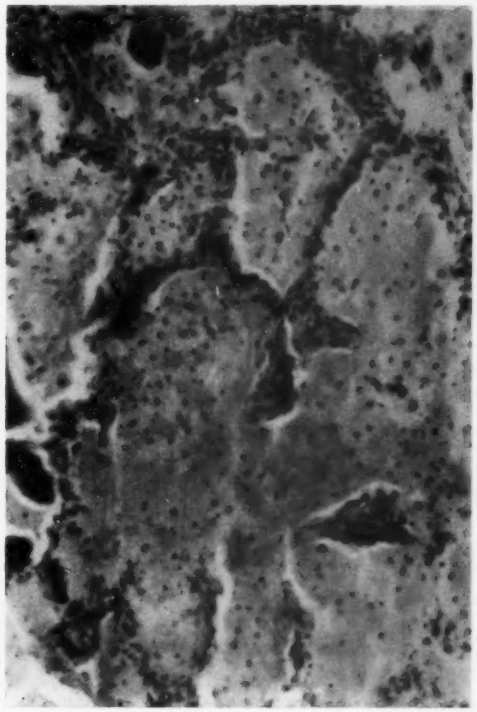

FIGURE VIII

Photomicrograph showing large histiocytes lying within the alveoli and merging into the structureless alveolar plug. (Hæmatoxylin and eosin stain, ×160.)

The Bronchioles. The walls and peribronchial tissues are normal. The lumina contain the same inspissated material mixed with blood as seen in the alveoli.

The Blood Vessels. Arteries and veins are normal. There is no evidence of thickening suggesting a pulmonary hypertension.

Special Stains.

(1) Lipoids. Unfortunately no tissue was preserved for frozen section. However sections were prepared for staining the alcohol insoluble

fats. No evidence of abnormal lipoid deposition was found. Specimens of sputum were also examined for lipoid stored in histiocytes. Good preparations were obtained but failed to show any evidence of lipoid material.

(2) Iron. Apart from occasional granules there is no iron-containing pigment shown. The brown granules in the histiocytes are presumably comprised of hæmatoidin or non-hæmatogenous particulate material.

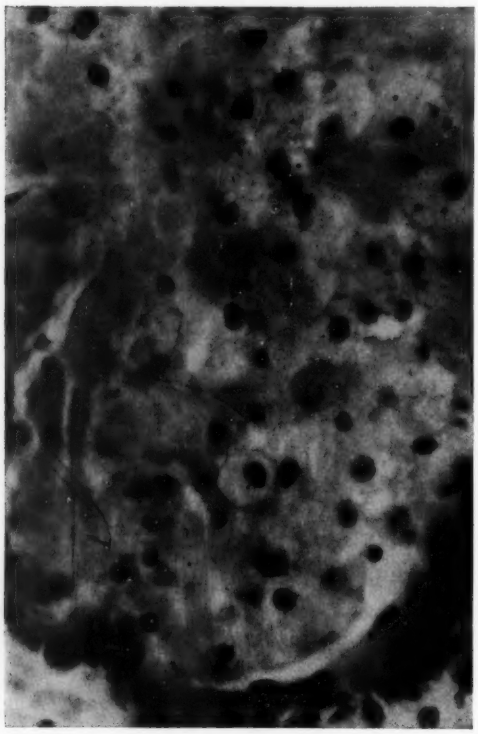

FIGURE IX

Photomicrograph showing large histiocytes lying within the alveoli and merging into the structureless alveolar plug. (Hæmatoxylin and eosin stain, ×400.)

(3) Phosphotungstic Acid-Hæmatoxylin. This stain was applied in an attempt to show the nature of the intra-alveolar material. It appears as a uniform, granular, red substance with occasional blue erythrocytes. There is no evidence of fibrin threads.

Comment. The histological findings do not point to any of the common pulmonary pathological conditions. Neoplastic and inflammatory diseases may be excluded if the biopsy specimen is representative of the lung tissue generally. The condition is essentially a passive, non-inflammatory accumulation of inspissated granular material in the alveoli. The precise nature of this material is in doubt but it appears to consist of cell debris and protein.

The essential histological features of this case are shown in the photomicrographs reproduced (Figures VI to IX). Figure VI gives a low-power view of the alveolar bed filled with the protein-like material, without evidence of intra-alveolar or interstitial inflammation. Figure VII shows a bronchiole filled with the same material. Figures VIII and IX, of higher magnification, show the large histiocytes lying within the alveoli and merging into the structureless alveolar plug. In retrospect, it is apparent that the large histiocytes of the original report are the " septal " cells of Rosen, Castleman and Liebow.

DISCUSSION

We were, then, left with a patient completely recovered from an obscure and life-threatening illness with gross radiographic and pathological changes which were unique in our experience. In July, 1958, our attention was drawn to the recently published article by Rosen, Castleman and Liebow freely quoted above. Further study of this article, and a review of the data relating to our own case, prompted us to submit a detailed case record, radiographic transparencies and histological preparations to Dr. Averill L. Liebow for his consideration. Dr. Liebow, in a letter dated August 15, 1958, remarks *inter alia* that " the histological features are identical with those of our cases ". He encouraged us still further by expressing the hope that we would report our case, not only because it would extend geographic and racial boundaries of this disease or group of diseases, but also because it might point the way to an effective treatment.

REFERENCE

ROSEN, S. H., CASTLEMAN, B., and LIEBOW, A. L. (1958), " Pulmonary Alveolar Proteinosis ", *New Engl. J. Med.*, **258**, 1123.

POSSIBLE SITES OF MACROGLOBULIN SYNTHESIS: A STUDY MADE WITH FLUORESCENT ANTIBODY[1]

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With an Addendum by

J. F. O'DEA
From the Commonwealth Serum Laboratories, Melbourne

SUMMARY

This paper describes experiments on the localization of macroglobulins by the fluorescent antibody technique in the tissues of two patients, one showing Mikulicz's syndrome and the other suffering from myeloma.

The first patient had the macroglobulin in large quantities in mature and immature plasma cells in the bone marrow. It was considered that the macroglobulin was a product of a plasmacytosis reactive to the pathological process in the patient's parotid gland.

The second patient had both a macroglobulin and a myeloma globulin in his serum. The fluorescent antibody studies suggested that these two abnormal proteins originated in different plasma cells in the bone marrow, which were, however, morphologically identical with orthodox staining. Neither protein could be demonstrated in frozen sections of other tissues obtained at autopsy.

WALDENSTRÖM (1948) first used "macroglobulin" to describe proteins in human serum having molecular weights of over 1,000,000. In his cases these proteins were associated with a clinical syndrome characterized by lethargy, bleeding tendency, hæmorrhagic purpura and enlargement of the liver, spleen and lymph nodes. The bone marrow was often invaded by so-called lymphocytoid cells, many of which showed cytoplasmic irregularity or loss. In smaller amounts, macroglobulins have been found associated with lymphosarcoma, and, very rarely, myeloma.

Recently there has been interest in the possibility of obtaining direct evidence that these unusual proteins may be found in the abnormal cells seen in such conditions (and in dysproteinæmias generally). Wollensak and Seybold (1956) used the fluorescent antibody technique of Coons and Kaplan (1950) to demonstrate abnormal serum globulins in the bone-marrow plasma cells in cases of myeloma, and Vasquez (1958) similarly demonstrated in these cells antigens cross-reacting with normal gamma globulin.

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Because of the range of abnormal cell types found in the bone marrow and other tissues in cases of macroglobulinæmia, a similar study of this condition is thought to be of interest. This paper describes experiments on the localization of macroglobulins by the fluorescent antibody technique in the tissues of two patients, one showing Mikulicz's syndrome and the other suffering from myeloma. The serum of both patients contained considerable amounts of macroglobulin (over two grammes per 100 millilitres in each case), although neither showed the clinical signs associated with macroglobulinæmia by Waldenström (1948, 1952).

CASE REPORTS

Patient A, a man, aged 51 years, gave a history of anæmia of two and a half years' duration, and of swelling in the region of the parotid and submaxillary salivary glands and the lachrymal glands. His spleen was palpable three centimetres below the left costal margin. Microscopic examination of sections of tissue obtained by parotid biopsy revealed replacement of the acinar tissue by small lymphocytes, surviving ducts and ductules being seen amongst the lymphoid tissue. Examination of peripheral blood film revealed 7% plasma cells and 7% lymphocyte-like plasma cells. The bone marrow was of normal cellularity, apart from the presence of a larger than usual number of plasmacytes (5%) and proplasmacytes (5%) (Figure I).

Electrophoresis of the serum (Figure II) revealed a high gamma peak, representing a concentration of 3·2 grammes per 100 millilitres. Examination in the ultracentrifuge (see addendum) showed a component with sedimentation constant of 15·8 Svedberg units. Nearly all the material under the high gamma peak could be precipitated from the serum on fivefold dilution with distilled water. This is a characteristic of macroglobulins.

A diagnosis was made of Mikulicz's syndrome. The underlying disease being regarded as probably lymphosarcoma, the patient was given three intravenous injections of P_{32} totalling six microcuries over a period of 20 days. During the next six weeks the enlarged glands gradually subsided, and there was a fall in the number of plasma cells in the peripheral blood. There was also some diminution in the serum macroglobulin concentration, with a slight reduction in the number of plasmacytes and proplasmacytes in the bone marrow. The marrow for fluorescent antibody studies was obtained 12 weeks after the last injection of P_{32}. At the time of writing (18 months after first observation), the patient has had a recurrence of parotid gland swelling and anæmia. The peripheral blood now contains 7% of plasma cells, although the level of macroglobulin in his serum is low (0·6 grammes per 100 millilitres). Some splenic enlargement persists. At no time has the patient shown any lymph-node enlargement.

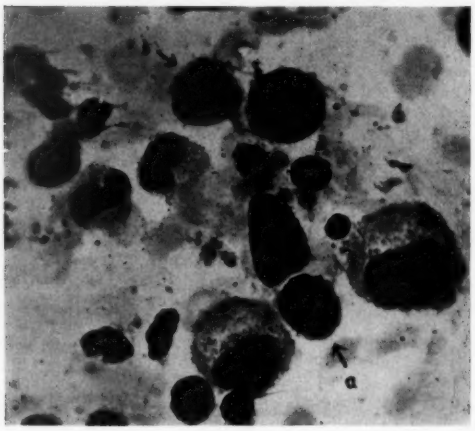

FIGURE I

Bone marrow smear of Patient A., stained with hæmatoxylin and eosin; (*a*) plasmacyte; (*b*) proplasmacyte.

Patient B, a man, aged 49 years, was suffering from advanced myeloma, and radiological examination revealed multiple osteolytic lesions in the ribs, pelvis and upper parts of the femora. Bone marrow biopsy showed normoblastic, but greatly reduced, erythropoeisis and large numbers of plasma cells (Figure III). Electrophoresis of the serum (Figure IV) showed a typical myeloma pattern. The abnormal peak in the beta-gamma globulin region represented a protein concentration of 12 grammes per 100 millilitres. It was found, however, that approximately 15% of the material in this peak could be precipitated by diluting the serum fivefold with distilled water; this suggested the presence of macroglobulins. Examination of the serum in the ultracentrifuge showed the presence of a globulin with a sedimentation constant of 15 Svedberg units (see addendum).

The patient died in hospital 18 days after admission. Autopsy revealed very severe pneumonia and a prepyloric ulcer of the stomach, and confirmed the presence of multiple myeloma.

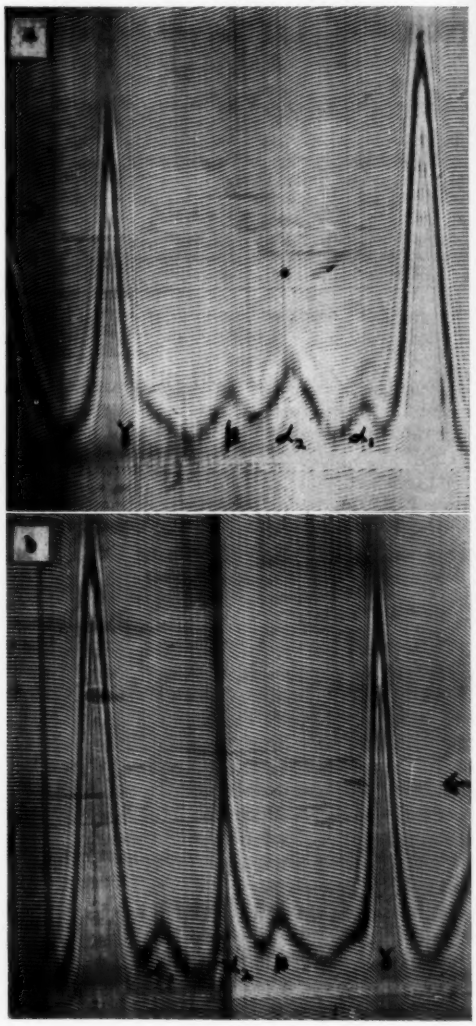

FIGURE II

Electrophoretic patterns of the serum of Patient A. Moving boundary electrophoresis in "Veronal" buffer (pH 8·6, I=0·1) for 5000 sec. at 25 volt cm.$^{-1}$ and 2°C. Total protein concentration in cell 0·8 gramme *per centum*. Phase-plate gradient curve with simultaneous Rayleigh interferometer pattern. Arrow and vertical line indicate direction of migration and origin. Above, ascending boundary; below descending boundary

MATERIALS AND METHODS

Buffers and Diluting Fluids

The buffers and diluting fluids used were as follows:

pH 8·6, I=0·1 "Veronal" buffer: 0·01520 M "Veronal", 0·1000 M "Sodium Veronal".

pH 7·2 phosphate buffered saline: 0·0048 M NaH_2PO_4, 0·0152 M Na_2HPO_4, 0·14 M NaCl.

pH 6·8, I=0·1 phosphate buffer: 0·04435 M NaH_2PO_4, 0·05565 M Na_2HPO_4.

pH 3·0 glycine buffer: 0·08 M glycine, 0·08 M NaCl, 0·02 M HCl.

Physiological saline: 0·155 M NaCl.

Electrophoresis

Moving boundary electrophoresis was carried out at 2°C. in a Perkin-Elmer Model 38A Tiselius apparatus fitted with a phase plate-gradient interferometric optical system (Moore and Oppermann, 1956).

Antigen-Antibody Titrations

Antibody activities were titrated by the Medical Research Council ring test (M.R.C., 1931). Antigenic specificity was studied by the technique of double diffusion in agar (Ouchterlony, 1953).

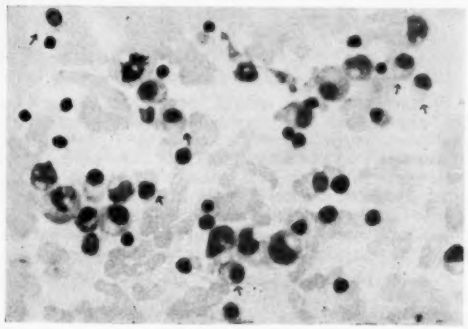

FIGURE III

Bone-marrow smear of Patient B., stained with hæmatoxylin and eosin. Plasma cells indicated by arrows

Purification of Antigens

Macroglobulin A was separated by diluting the serum of patient A with six volumes of distilled water. The precipitated macroglobulin was recovered by centrifugation at 1500 g. for 20 minutes at 0° C. On electrophoresis in pH 8·6, I=0·1, "Veronal" buffer, this material was found to contain two components with mobilities and concentrations respectively of $1·70 \times 10^{-5}$ volt^{-1} sec.$^{-1}$ cm.^{2} and 85%, and $2·92 \times 10^{-5}$ volt^{-1} sec.$^{-1}$ cm.^{2} and 15 %. When the current was reversed, the boundary representing the major, slow component remained sharp, whilst the minor boundary spread considerably. This indicated that the latter had a high diffusion constant and was not macroglobulin. Preparative separation of the two components was achieved by electrophoresis on starch blocks, after the method of Kunkel (1954). "Veronal" buffer, pH 8·6, I=0·1, and a potential gradient of 15 volt cm.$^{-1}$ for 16 hours were used.

Macroglobulin B was isolated from the serum of patient B by precipitation with distilled water and final purification by starch block electrophoresis as described above. It had a mobility of $2·01 \times 10^{-5}$ volt^{-1} sec.$^{-1}$ cm.^{2} on free electrophoresis in the pH 8·6 "veronal" buffer.

The myeloma protein was isolated by concentrating the supernatant fluid from the distilled water precipitation of macroglobulin B and submitting it to

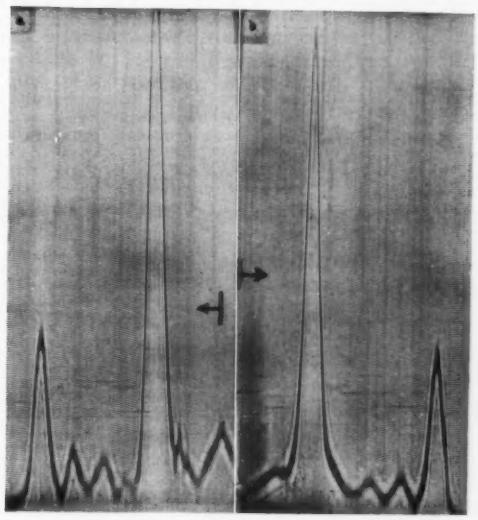

FIGURE IV

Electrophoretic patterns of the serum of Patient B. Moving boundary electrophoresis in "Veronal" buffer (pH 8·6, I=0·1) for 5000 sec. at 25 volt cm.$^{-1}$ and 2°C. Total protein concentration in cell 1·5 grammes *per centum*. Phase-plate gradient curve with simultaneous Rayleigh interferometer pattern. The arrow and vertical line indicate the direction of migration and origin. Left, ascending; right, descending

starch block electrophoresis under the same conditions as those used for the macroglobulins. It had a mobility of $1·87 \times 10^{-5}$ volt^{-1} sec.$^{-1}$ cm.^{2} on free electrophoresis in the pH 8·6 "veronal" buffer.

Gamma–2 globulin was prepared by low temperature ethanol fractionation of fresh human plasma by the method of Deutsch, *et alii* (1946).

Preparation of Antisera

Three 20 milligramme doses of the antigens were injected with Freund's adjuvant into the thighs of six months old rabbits at intervals of seven days. Three weeks after the last injection, the rabbits were bled from the marginal ear vein. The antibody containing globulin was isolated and concentrated by low-temperature ethanol fractionation (Nichol and Deutsch, 1948). Each antiserum reacted to high titre with its specific protein and also cross-reacted with the other two proteins and with normal human gamma—2 globulin (Figures Va and Vb). Highly specific antibodies were obtained by passing each purified globulin fraction

through a column of azo-benzyl cellulose coupled to its cross-reacting antigens, after the method of Campbell *et alii* (1951).

Preparation of Fluorescent Antibody

Fluorescein isocyanate was prepared and conjugated to the antibody globulin fractions by the methods of Coons and Kaplan (1950). Diffusible fluorescein derivatives were removed, and the conjugated protein was concentrated by electrodialysis in pH 6·8, I=0·1 phosphate buffer, at 2°C. for 48 hours in an electrophoresis convection apparatus constructed after the design of Raymond (1952).

Elimination of Non-Specific Staining.—The most serious complication in the use of fluorescein-conjugated antibody is the presence of non-specifically-staining fluorescent material in the conjugates, even after exhaustive dialysis. Coons and Kaplan (1950) recommended the absorption of the conjugates with acetone-dried powders of various tissues, notably rat liver. This treatment suffices to eliminate non-specific staining of most tissues, except bone marrow. In human bone marrow, fluorescein-globulin conjugates give a brilliant non-specific fluorescence with the eosinophil cells. Absorption with acetone-dried rabbit bone-marrow powder (Sheldon, 1953) or dog-spleen powder diminished the reaction slightly. Finally, it was found that the non-specific staining could be eliminated by adsorbing the antibody to its antigen coupled to azobenzyl-cellulose in a column and eluting it in pH 3·0 glycine buffer. The conditions were as follows. The antigen-coupled azobenzylcellulose was packed into a column 0·5 × 10 centimetres in size, and a 1% solution of the conjugated globulin in pH 6·8 phosphate buffer was run through it until the filtrate contained traces of antibody (titre 1/20 as determined by the ring test). The column was then washed with 50 millilitres of the phosphate buffer. The antibody free of the non-specifically staining substance was eluted by passing 5 millilitres of the pH 3·2 glycine buffer through the column. The eluate was dialyzed for 48 hours against two changes of pH 7·2 phosphate-buffered saline. The solution was still fluorescent, and 96% of the protein (as measured by ultra-violet absorption at 270 mμ) was precipitated by the specific antigen. The recovery of antibody activity was approximately 30%.

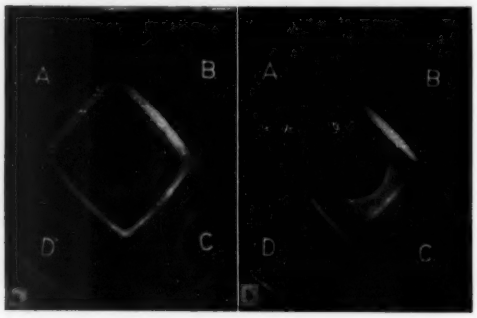

FIGURE V

Ouchterlony prints showing immunological relationships between macroglobulin A, macroglobulin B, the myeloma protein and normal gamma—2 globulin. (*a*) Centre well contains anti-macroglobulin A; well A, macroglobulin A; well B, gamma—2 globulin; well C, gamma—2 globulin; well D, myeloma protein. (*b*) Centre well contains anti-myeloma protein; well A, macroglobulin B; well B, myeloma protein; well C, macroglobulin A; well D, gamma—2 globulin

Preparation of Smears and Sections

Bone marrow was obtained by sternal puncture, and smears were made on slides which had been previously dipped in 1% Armour bovine serum albumin and dried at 75°C. Studies with fluorescent antibodies were preferably made within four hours of taking the marrow. If the smears had to be left, they were plunged into isopentane at —60°C. and stored at this temperature.

In the case of Patient B, small (0·1 to 0·5 grammes) pieces of liver, lymph node, spleen and muscle were obtained at *post mortem*, quickly frozen in isopentane at —60°C. and stored at —20°C. Frozen sections (6 to 8 μ) were cut at —20°C. by the use of a Cambridge rocking microtome cooled with dry ice and fitted with the microtome knife attachment described by Coons *et alii* (1951). The sections were dried in a draught of air at 2°C. and immersed in 95% aqueous ethanol for ten minutes at 37°C.

Application of Fluorescent Antibody

Fluorescent antibody was applied to the marrow smears and tissue sections by the methods of Coons and Kaplan (1950). Photographs were taken within two to three hours of staining.

Fluorescence Microscopy

A 2000 watt direct current carbon arc was used as the light source. The primary filter was a 4 centimetre thickness of a solution of 4 grammes of copper sulphate in 100 millilitres of ammonia (50%). A non-fluorescing condenser (N.A.1.25) was used, and the secondary filter, a double thickness of Wratten 15 yellow, was inserted in the ocular. The microscope, primary filter and light source were rigidly mounted on an optical bench to minimize vibration during photography.

Photographs were taken on Kodak "Tri-X" 35 millimetre film. Exposure times at the highest magnifications (×650) averaged 15 minutes. The films were developed for 12 minutes at 20°C. in May and Baker "Promicrol" developer.

Conventional Staining after Application of Fluorescent Antibody

Coons *et alii* (1955) mentioned that conventional staining after the application of fluorescent antibody was rarely successful. In this work it was found possible to stain marrow smears with hæmatoxylin-eosin if the minutes of incubation with fluorescent antibody were reduced from the 30 recommended by Coons to one. This reduced the intensity of the fluorescence, and increased the photographic exposure time to 35 minutes. It was found possible to treat the marrow of Patient B sequentially with fluorescent anti-macroglobulin, fluorescent anti-myeloma protein and hæmatoxylin-eosin.

RESULTS

Observations on Patient A

The cytoplasm of the bone-marrow plasma cells of Patient A fluoresced strongly when treated with fluorescent anti-macroglobulin A,

indicating the presence of large amounts of macroglobulin A (Figure VI).

Bright fluorescence was also observed in the peripheral cytoplasm of many cells which, on conventional staining, were found to be proplasmacytes or plasmablasts. Marrow

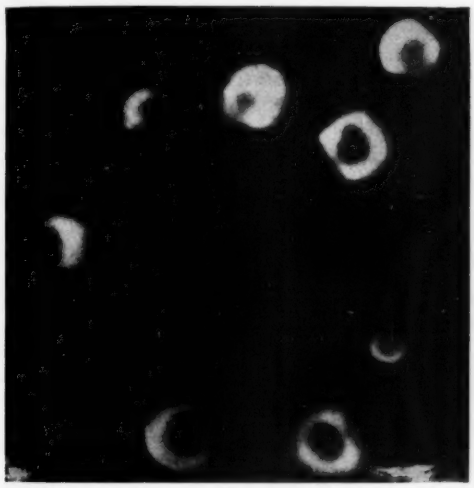

FIGURE VI

Fluorescence micrograph of bone-marrow smear of Patient A, treated with fluorescent anti-macroglobulin A, showing (*a*) plasmacytes and (*b*) proplasmacytes. The cytoplasm in each cell in the original appeared to fluoresce uniformly

smears previously incubated with preparations of high-titre anti-macroglobulin A did not react with the fluorescent antibody. This indicates that the staining was specific (Coons and Kaplan, 1950). None of the cells in the marrow smear reacted with macroglobulin-absorbed fluorescent anti-gamma-2 globulin.

Observations on Patient B

Application of fluorescent anti-macroglobulin B to a bone marrow smear of Patient B produced fluorescence in a number of plasma cells (Figure VIIa). The smear was washed free of buffered glycerol with phosphate-saline and incubated with fluorescent anti-myeloma protein, and the same field was observed under the fluorescence microscope. More plasma cells were observed to fluoresce (Figure VIIb). In another experiment the order of application of the fluorescent antibodies was reversed. In this case it was possible to find fields where the application of fluorescent anti-macroglobulin B caused a small increase in the number of plasma cells fluorescing after treatment with fluorescent anti-myeloma protein. This suggested that the macroglobulin and myeloma protein were present in different plasma cells. In smears stained with hæmatoxylin-eosin after these experiments there were often a few plasma cells which had not reacted with either antibody. The counts of plasma cells observed in these experiments are given in Table I. Frozen sections of Patient B's tissues failed to react with either fluorescent anti-myeloma protein or anti-macroglobulin B. Sections of spleen (Figure VIII) and lymph node did, however, react with fluorescent anti-gamma-2 globulin.

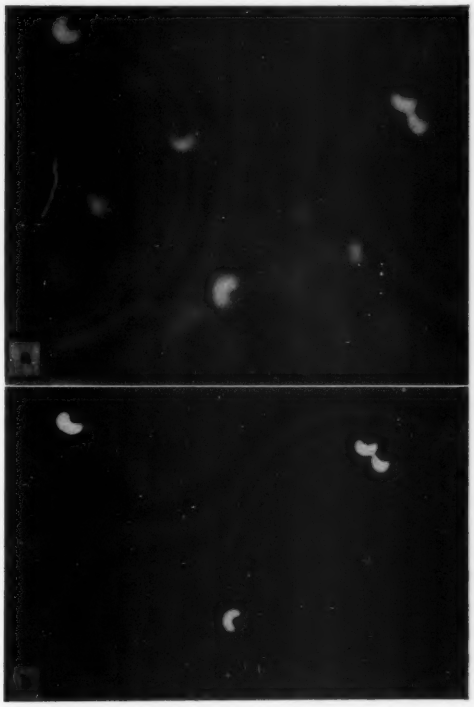

FIGURE VII*a*

Fluorescence micrograph of bone-marrow smear of Patient B, treated with fluorescent anti-macroglobulin B, showing fluorescing plasma cells. The heavy background of this micrograph is due to fluorescing rouleaux of erythrocytes, which were apparently coated with an appreciable amount of macroglobulin

FIGURE VII*b*

Same field as shown in Figure VII*a*, after treatment of the slide with fluorescent anti-M protein

Pre-treatment of the marrow smears with high-titre anti-myeloma protein led to lack of staining with the corresponding fluorescent antibody, although the reaction with fluorescent

anti-macroglobulin B was normal. Similarly pre-treatment with fluorescent anti-macroglobulin B suppressed the reaction with fluorescent anti-macroglobulin, but not with fluorescent anti-myeloma protein. This indicates that the staining observed was specific.

TABLE I

Counts of Plasma Cells after Serial Treatment of Bone-Marrow Smears of Patient B with Fluorescent Anti-Macroglobulin B, Fluorescent Anti-Myeloma Protein and Hæmatoxylin-Eosin

Order of Treatment	Count[1]
1. Fluorescent anti-macroglobulin B	2·5 (1·1)
2. Fluorescent anti-myeloma protein	7·3 (1·9)
3. Hæmatoxylin-eosin	8·1 (1·7)
1. Fluorescent anti-myeloma protein	5·9 (2·1)
2. Fluorescent anti-macroglobulin B	7·9 (2·2)
3. Hæmatoxylin-eosin	8·4 (1·5)

[1] Each count is the mean of 20 fields. The figure in parentheses is the standard deviation.

DISCUSSION

There is a similarity between the observations made on the marrow of Patient A and those reported by Ortega and Mellors (1957) on the localization by fluorescent antibody of normal gamma-globulin in human splenic and lymphatic tissue. Ortega and Mellors observed that gamma-globulin was abundant in plasmacytes

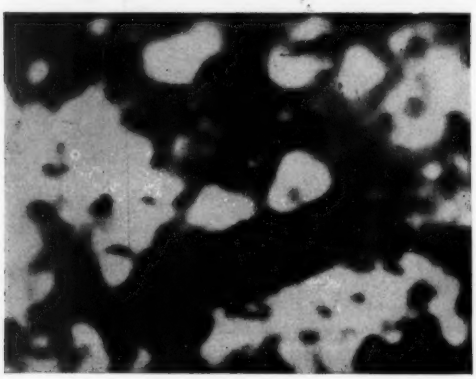

FIGURE VIII

Fluorescence micrograph of a section of the spleen of Patient B., treated with fluorescent anti-gamma—2 globulin

and proplasmacytes. In an experimental study of antibody production in the rabbit, Coons, *et alii* (1955) observed that after an antigenic stimulus the first cells with demonstrable antibody were large, with a thin rim of basophilic cytoplasm, resembling in every way primitive hæmatogenous cells. They considered that these cells actively synthesized antibody as they multiplied and differentiated to mature plasma cells. Many of the immature cells observed in the marrow of Patient A appeared to be proplasmacytes, containing high concentrations of macroglobulin in their cytoplasm. Although it is not possible to follow the synthesis of macroglobulin after a specific stimulus, it seems reasonable to suppose, in analogy with the results of Ortega and Mellors with gamma-globulin, that these proplasmacytes represent varying stages in the differentiation and maturation of the macroglobulin-containing plasma cells. Like normal gamma-globulin, macroglobulin may be synthesized during the development of the plasma cell from the plasmablast.

When first observed, the bone marrow picture and the presence of plasma cells of varying degrees of maturity in the peripheral blood were suggestive of a reactive plasmacytosis stimulated by the pathological process in the parotid glands. It is possible, therefore, that Patient A had a reactive macroglobulinæmia. It is unfortunate that no material from biopsy of the parotid glands was available for fluorescent antibody studies, which could strengthen or weaken this supposition. Reactive dysproteinæmia in inflammatory conditions and malignant disease has occasionally been reported in the literature. Bohrod (1957) reported peripheral blood and bone marrow plasmacytosis and cryoglobulinæmia in a patient with adenocarcinoma of the breast. Plasmacytosis with uncharacterized hyperglobulinæmia has been observed in some other malignant conditions (Clark and Muirhead, 1954) and in Hashimoto's syndrome (Beare, 1958). Mackay (1956) has suggested that macroglobulins are synthesized by malignant lymphocytic cells as a result of a somatic mutation in the same cell line as that from which these cells originated. The results obtained with Patient A suggest that macroglobulins need not always be produced in this manner, but may sometimes arise from a reactive process.

The results of the study of Patient B suggest that the myeloma-protein and macroglobulin B were being produced by different plasma cells in the marrow, which were, however, morphologically identical with orthodox staining. This could have been more convincingly demonstrated had a second fluorescent label been available. Recently, Chadwick, *et alii* (1958) have described the use of the dye Lissamine-Rhodamine, R.B. 200 as a distinctive alternative label to fluorescein. It seems that this could be profitably employed as a counter-stain in future studies of mixed dysproteinæmias.

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Patient A was admitted to the Peter MacCallum Clinic, Melbourne, under Dr. J. Madigan. Patient B was admitted to the Alfred Hospital, Melbourne, under Professor R. R. H. Lovell. The writer is indebted to Dr. R. Motteram, pathologist to the Peter MacCallum Clinic, for access to material from Patient A, and to Dr. A. V. Jackson, director of pathology at the Alfred Hospital, for material from Patient B. The writer is also indebted to Dr. R. Motteram, and to Dr. R. A. Hayes, deputy-director of pathology at the Alfred Hospital, for discussions and advice concerning the histological findings in both cases. Mr. T. O'Connor, photographer to the Alfred Hospital, prepared the prints for the illustrations.

ADDENDUM

Ultracentrifugal Studies

(J. F. O'D.)

Ultracentrifugal studies were performed in a "Spinco Model-E" ultracentrifuge at various dilutions in 0·2 M NaCl solution. Typical ultracentrifugal patterns and experimental conditions are shown in Figures IX and X.

Patient A.—In the serum diluted to one-half with 0·2M saline, the fast macroglobulin component was followed by an asymmetrical peak corresponding to the residual globulin and albumin (Figure IX). When the serum was diluted to one-quarter in 0·2M saline, a sedimentation constant, corrected relative to water at 20°C. (S_{20}, w) of 15·8 was found for the fast-moving component.

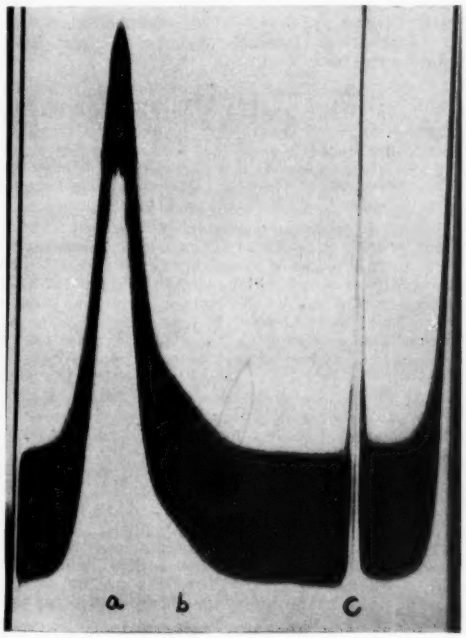

FIGURE IX

Ultracentrifuge pattern of serum of Patient A. Direction of sedimentation in pattern from right to left. Photograph taken 98 minutes after reaching top speed of 50,720 revolutions per minute. Mean temperature 15·0°C. Serum diluted to half in 0·2 M saline. (*a*) Albumin peak; (*b*) normal globulins; (*c*) macroglobulin

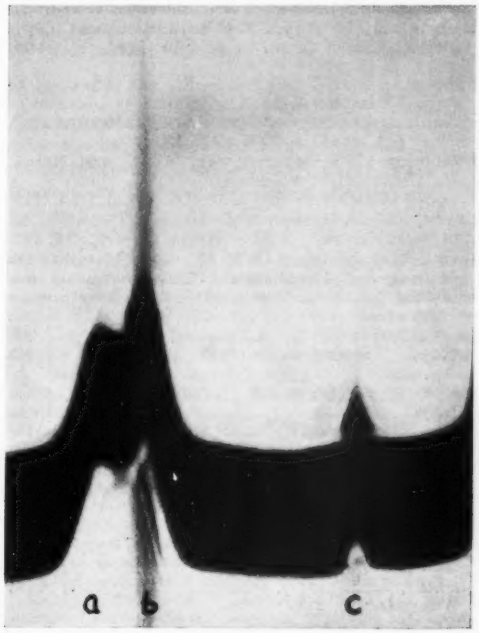

FIGURE X

Ultracentrifuge pattern of serum of Patient B. Direction of sedimentation in pattern from right to left. Photograph taken 42 minutes after reaching top speed of 59,760 revolutions per minute. Mean temperature 20·9°C. Serum diluted to one-fifth with 0·2 M saline. (*a*) Albumin; (*b*) normal globulins and myeloma protein; (*c*) macroglobulin

Patient B.—In the serum diluted to one-fifth with 0·2M saline, the fast macroglobulin component was followed by a double peak corresponding to globulin and albumin (Figure X). The shape of this double peak indicates a markedly elevated globulin-albumin ratio. When the serum was diluted to one-seventh with 0·2M saline, a sedimentation constant, corrected relative to water at 20°C. (S_{20}, w) of

15·0 was found for the fast-moving macroglobulin component.

Acknowledgement.—I wish to acknowledge the technical assistance of Mr. G. Wilson.

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ESSENTIAL CRYOGLOBULINÆMIA AND ACQUIRED HÆMOLYTIC ANÆMIA DUE TO COLD AGGLUTININS[1]

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SUMMARY

A case of hæmolytic anæmia associated with a cold agglutinin and essential cryoglobulinæmia is described. The patient did not respond to steroid therapy, but his condition was greatly improved by protection against the cold. Immunological and electrophoretic studies showed that two abnormal proteins were present. One was a cold agglutinin immunologically identical with gamma globulin, and the other was a cryoglobulin which was unrelated immunologically to any normal serum protein.

ESSENTIAL CRYOGLOBULINÆMIA and acquired hæmolytic anæmia due to cold agglutinins are both rare, but their simultaneous occurrence has been reported in some patients (Conn, 1955; Christensen and Dacie, 1957). The present report concerns immunological studies of the abnormal proteins present in a patient who had both conditions.

CASE REPORT

The patient, J.T.C., was a white, single male, aged 73 years, who until nine years before had been employed as an engineer. There was no relevant family history, and he had enjoyed good health apart from a gonorrhœal infection in 1904 and a prostatectomy in 1940.

In 1946 he first experienced attacks of Raynaud's phenomena in all extremities, some of which preceded the passage of red urine. In recent years these episodes were followed by symptoms of anæmia, and in July, 1954, he was admitted to another hospital with severe anæmia and lobar pneumonia. While in hospital, he was given a transfusion of three litres of blood. For several years he had noted an increasing tendency to bruise after minor trauma, and he had also experienced angina of effort.

He was first seen by one of us (G.A.W.J.) on his admission to Sydney Hospital in October, 1955, at which time he had recurrent colicky abdominal pain, which was precipitated by cold drinks. The pain began in the right iliac fossa and radiated towards the left side of the abdomen, and was similar to previous attacks, which had been followed by the passage of red urine.

Physical examination showed him to be a thick-set, elderly man with a senile cataract in the left eye. Mucosal pallor, scattered bruises on his limbs and enlarged left axillary and right epitrochlear lymph nodes were found. His liver was seven centimetres and his spleen three centimetres below the costal margin, and both organs felt smooth and were not tender. The apex beat was two centimetres outside the mid-clavicular line, and a blowing aortic systolic murmur was heard, which was propagated down the left side of the sternum. Investigation of the urine gave normal results, and a full systemic examination revealed no other abnormal findings.

The hæmoglobin value on 21st October, was 11·4 grammes per 100 millilitres, the venous hæmatocrit reading was 29%, the leucocytes numbered 6000 per cubic millimetre and had a normal distribution, and the platelets numbered 150,000 per cubic millimetre. The erythrocyte sedimentation rate (Westergren) was 60 millimetres per hour at room temperature. Examination of a stained film showed marked clumping of red cells, which were orthocytic and normochromic. Cold agglutinins were demonstrated, and the direct Coombs test produced a positive result at room temperature, but a negative result at 37°C. Leucagglutinins were also detected. A Donath-Landsteiner test produced a positive result and the Wassermann and Kahn reactions of the serum were negative. The hæmoglobin values on November 11 and 21 was 8·8 grammes per 100 millilitres and on November 28, 4% of the erythrocytes were reticulocytes. Examination of the sternal marrow aspirate showed "marked hyperplasia due to increased normoblastic erythropoiesis". The total serum protein content was 6·8 grammes per 100 millilitres (albumin 3·6, globulin 3·2 grammes per 100 millilitres) (Howe). Dr. H. S. H. Wardlaw reported the electrophoretogram as showing "a fraction between beta and gamma globulin which may be beta-2 or may indicate the presence of an abnormal group of gamma globulin". The presence of a serum cryoglobulin was demonstrated. The result of the direct Van den Bergh test was always negative, and serum bilirubin estimations varied from less than 0·5 milligramme per 100 millilitres on 7th November, to 2·7 milligrammes on 9th December. The thymol turbidity was two units and the serum alkaline phosphatase level was 11·6 King-Armstrong units. The blood urea nitrogen content was 18 milligrammes per 100 millilitres and the blood creatinine content 1·0 milligramme per 100 millilitres. The electrocardiogram was within normal limits. Microscopic examination of the urine on 12th November, revealed 4 to 10 leucocytes, 2 to 6 red cells and an occasional granular cast per high-power field, but no organisms were grown on culture. From November 23 to 28 urine specimens were examined daily for

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[5] Supported in part by the National Health and Medical Research Council of Australia.

hæmoglobin, always with negative results. An X-ray examination of the chest revealed some pleural thickening in the left costo-phrenic angle. No other lesion was seen in the lung fields. The cardiac outline was hypertensive in type. A Graham's test did not reveal any gall-stones, and the gall-bladder appeared to function normally.

A diagnosis of acquired hæmolytic anæmia (cold-agglutinin type) and essential cryoglobulinæmia was made, and the patient was transfused with packed cells and steroid therapy was commenced. ACTH, in a dosage of 80 units per day, was given for two weeks, and his condition improved. He was discharged from hospital on December 24, taking 75 milligrammes of cortisone per day. He was maintained on this dosage for eight months, during which time his hæmoglobin value varied from 8·5 to 10·9 grammes per 100 millilitres, and a reticulocyte count performed on June 1, 1956 gave a figure of 7·5%. His general condition was not improved by the steroid therapy, nor had there been any decrease in the cryoglobulin level (Table I), so that when he was readmitted to hospital on August 27th, 1956, with another severe bout of abdominal pain, cortisone therapy was suspended. On this occasion the physical findings were similar to those on the first admission. A surgical consultation was obtained, and a diagnosis of renal colic was suggested. Laboratory tests gave the following results. The hæmoglobin value was 10·4 grammes per 100 millilitres, 3·7% of the erythrocytes were reticulocytes, and the prothrombin index was 100%. The erythrocyte sedimentation rate (Westergren) was 88 millimetres in one hour at room temperature and 18 millimetres in one hour at 37°C. Examination of sternal marrow revealed increased cellularity due to erythropoietic hyperplasia. The polychromatic normoblast was the predominant cell, whilst granulopoiesis and platelet production appeared normal. Plasma cells were infrequent and did not show vacuolation. The cold agglutinin titre of whole serum was 1 : 8,000,000 (Table II). An X-ray examination of the skull revealed no destructive bone lesion. An X-ray examination of the lower thoracic part of the spine revealed well-marked degenerative lipping of the vertebral bodies, with fusiform dilatation of the aorta opposite the second, third and fourth lumbar vertebræ. In an intravenous pyelogram taken on August 17th, the only abnormality seen was a double collecting system on the right side. No radio-opaque calculi were seen in the plain X-ray film.

In view of the negative radiological findings, it was thought that the abdominal pain was part of the primary disease process, and no specific therapy was given apart from bed rest and warmth. The abdominal pain disappeared, and he was discharged from hospital on September 19th.

The patient was kept under regular observation, and on November 9th, 1956, coagulation studies gave normal results (Table III). A red cell survival test (Mollison, 1956) commenced on November 30th, showed 53% of Cr^{51} remaining in the circulation after eleven days, and the survival curve gave an approximate red-cell life of 80 days.

During the first half of 1957 his general health remained satisfactory, and except for angina of effort he was symptom-free. Hæmoglobin values varied between 9·3 and 10·6 grammes per 100 millilitres, and the percentage of reticulocytes between 2·8 and 4·4. Although he attempted to keep warm at all times, he suffered another hæmolytic crisis, and was readmitted to hospital on July 11th, with weakness, pallor and angina of minimal effort. Again no specific therapy was given, and with warmth and rest his hæmoglobin value rose from 7·1 to 11·0 grammes per 100 millilitres within three weeks and he produced a maximum reticulocyte response of 14·8%. At this time the cold agglutinin titre of whole serum at 4°C. was 1/32,000 (Table II).

Since his discharge from hospital on August 9th, 1957, the patient has continued to feel well and his angina has been considerably relieved by "Prænitron" tablets; the latest investigations, performed on December 10th, 1957, showed that the cryoglobulin level was unchanged, and that the cold agglutinin titre in whole serum at 4°C. was 1/500,000. The direct Coombs test produced a positive result with cells maintained at 37°C. and with cells which had remained in contact with patient's serum for 30 minutes at 4°C. In the former test the result was weakly positive at a dilution of human globulin antiserum of 1 in 32, and in the latter it was strongly positive at 1 in 32. No L.E. cells or any other phagocytosed material was found in preparations made by defibrinating blood at 37°C. and at 4°C. The Hess test produced a positive result. The application of an ice cube to his abdomen for 60 seconds did not produce any effect.

METHODS OF INVESTIGATION

Separation of Cryoglobulin for Agglutination Titres

Cryoglobulin for Agglutinin Titres.—Venous blood was collected, allowed to clot and centrifuged at 37°C. The serum obtained was kept at 4°C. for 48 hours, and the supernatant was pipetted off and used as "serum without cryoglobulin". The cryoglobulin which remained was washed twice by dissolution in physiological saline at 37°C., the mixture being allowed to stand at 4°C. for 48 hours to reprecipitate the cryoglobulin and the supernatant saline being discarded. The resultant cryoglobulin was shown to be electrophoretically pure (Figure I).

Titration of Cold Agglutinins

The titre of cold agglutinins was estimated by serial dilutions of the test material with saline (Dacie, 1956). Readings were made macroscopically and confirmed microscopically, to eliminate the possibility of confusing agglutination and pseudoagglutination (Barr *et alii*, 1950).

The Cryoglobulin Level

The cryoglobulin level was estimated by the method of Mackay *et alii*, (1956), in which the amount of cryoglobulin in whole serum is measured in a Wintrobe hæmatocrit tube and expressed as a percentage termed the cryocrit (Table I).

Electrophoretic Studies

Paper electrophoresis[1] was carried out with the use of Whatman Number 1 paper between siliconed plates and a "Veronal"/"Veronal" buffer at pH 8·6 and ionic strength 0·05. The dried paper was stained for protein with bromphenol blue, and the periodic acid Schiff carbohydrate stain (Roboz *et alii*, 1955) and Sudan black lipid stain (Dangerfield and Smith, 1955) were used when indicated.

Immunological Studies

Rabbit antisera were prepared against the patient's cryoglobulin (isolated as described above, but washed three times in physiological saline), Bence-Jones

[1] Routine electrophoretic patterns during the patients' course were prepared by Dr. H. S. H. Wardlaw, using a slight modification of the method of Flynn and de Mayo (1951).

proteose, a macroglobulin, and various fractions of normal human serum by the technique of Kabat and Mayer (Firkin, 1958).

Albumin, alpha-2, beta and gamma globulins were obtained by eluting with isotonic saline the appropriately identified zones of paper electrophoretograms of normal serum (Wunderly *et alii*, 1953).

Ouchterlony's method of gel-diffusion (Oudin, 1952) was employed to study the immunological properties of the isolated cryoglobulin. Purified agar (supplied by Davis Gelatine (Australia) Proprietary Limited)

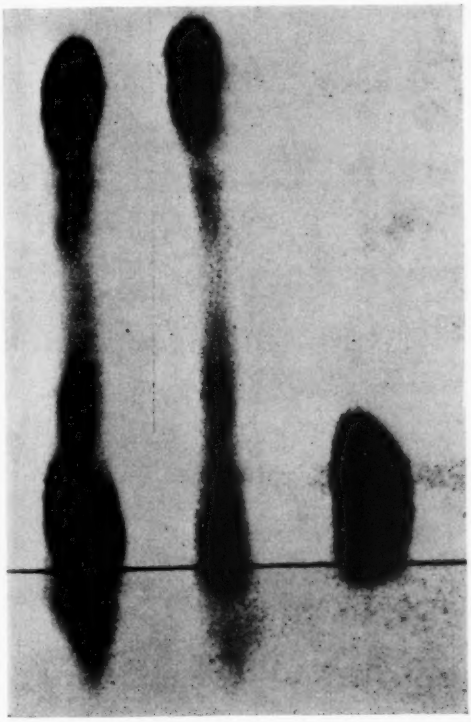

FIGURE I

Electrophoretic patterns of the patient's serum stained with bromphenol blue. On the left is the patient's whole serum, next the patient's serum after extraction of the cryoglobulin, and on the right the extracted cryoglobulin

was made up to a concentration of 1·5%weight for volume with isotonic saline, merthiolate being added as a preservative to a final concentration of 1/10,000, and the hot agar poured into Petrie dishes nine centimetres in diameter. A shallow cup was made in the centre of each dish, and two, three or more identical cups were placed so that their edges were on the segment of a circle 1·2 centimetres from the edge of the central cup and 0·8 centimetre from each other (Figure II). The immunological characteristics of the cryoglobulin were compared with those of other proteins and protein fractions (*vide supra*) by appropriate combinations of antisera and the isolated proteins in these cups (Figures II and III).

The cold agglutinin was also identified immunologically by the use of Ouchterlony's technique (Figure II) (Firkin and Blackburn, 1956). One millilitre of packed group O, Rh-negative red cells, washed three times with physiological saline, was suspended in nine millilitres of the patient's serum from which the cryoglobulin had been extracted. The mixture was left for 12 hours in a cold room at 10° C., and was

TABLE I

Cryoglobulin Content of the Serum

Before Steroid Therapy		During Steroid Therapy	
Date	Cryocrit	Date	Cryocrit
2/12/55	10%	16/12/55	10%
12/12/55	10%	17/12/55	11%
13/12/55	9·2%	18/12/55	9·5%
		21/12/55	10%
14/12/55	10%	18/ 5/56	13%

centrifuged at the same temperature and the supernatant serum was discarded. The cold agglutinin was then eluted by the method of Aubert and Brendemoen (1949).

Coagulation Studies

Routine coagulation studies were first performed on the patient's whole blood, serum and plasma in the standard manner. Estimation of the prothrombin time, the thromboplastin generation test and the Macfarlane dilution test for fibrinolysis (Firkin *et alii*, 1957) were then repeated on the patient's plasma and serum from which the cryoglobulin had been precipitated. The results were as follows : the coagulation

TABLE II

Cold Agglutinin Titres

Date	Test Substance	Temperature		
		37° C.	20 °C.	4° C.
18/ 9/56	Whole serum	Nil	1/256	1/8,000,000
7/57	Whole serum	Nil	1/1024	1/32,000
	Serum without cryoglobulin	Nil	1/64	1/2000
	Cryoglobulin	1/16	1/16,000	1/6,000,000
10/12/57	Whole serum	1/2		1/500,000
23/12/57[1]	Whole serum	<1/5		1/325,000
	Whole serum without cryoglobulin	<1/5		1/5000
	Cryoglobulin	1/5		1/1,300,000

[1] Using serum obtained on December 10, 1957 and stored at 5° C.

time (Lee and White) was six and a half minutes ; the bleeding time (Duke) was two and a half minutes ; clot retraction was normal ; the platelets numbered 165,000 per cubic millimetre ; the result of the prothrombin consumption test (Quick) was normal. Other findings are shown in Table III.

The effect on the fibrinogen level of extraction of the cryoglobulin from the patient's plasma was shown in the following manner. Two test tubes were stood in a water bath at 37° C. Ten millilitres of physiological saline, 1 millilitre of the patient's whole plasma and three drops of " Topical Thrombin " (Parke Davis) were added to one tube, and 10 millilitres of physiological saline, 1 millilitre of the patient's plasma from

which the cryoglobulin had been precipitated and three drops of "Topical Thrombin" (Parke Davis) were added to the other. The result is shown in Figure IV.

The patient's urine was examined for Bence-Jones proteose with the use of a Bence-Jones antiserum by the method of Collier and Jackson (1953).

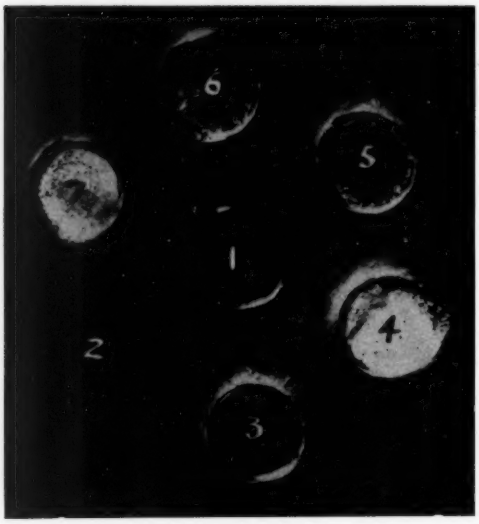

FIGURE II

Cup 1 contains human gamma globulin antiserum, cup 2 eluted cold agglutinin, cup 3 extracted cryoglobulin, cup 4 gamma globulin, cup 5 alpha-2 globulin, cup 6 albumin, and cup 7 beta globulin. A line of precipitate formed between cups 1 and 2 which ran into similar lines between cups 1 and 3 and cups 1 and 4, resulting in a reaction of identity. This shows that the cold agglutinin and the cryoglobulin extract both contain an antigen which is immunologically identical with gamma globulin

RESULTS OF INVESTIGATIONS

Cold Agglutinins

The titres of the cold agglutinin in the whole serum, the serum without cryoglobulin and the washed cryoglobulin extract are set out in Table II. There is a marked reduction in the titre of cold agglutinin in the serum after extraction of the cryoglobulin. The extreme variation in the titre of cold agglutinin (1/8,000,000 to 1/32,000) in the whole serum is difficult to explain, but is possibly related to the use of different red cells in these determinations.

The Cryoglobulins

The cryoglobulin precipitated as a transparent gel when the patient's serum or plasma was stood at temperatures below 15°C. The precipitate could be redissolved by warming in serum, plasma or saline to 37°C., and reprecipitated by cooling an indefinite number of times. Microscopic examination showed the precipitate to consist of amorphous material.

Electrophoresis

Paper electrophoresis of the patient's serum stained with bromphenol blue showed an abnormal protein, which migrated a little more slowly than beta globulin (Figure I). The intensity of the staining reaction of the cryoglobulin to the periodic acid Schiff reagent showed that the abnormal protein had a high glycoprotein content. The electrophoretic pattern when stained for lipids was normal, and on electrophoresis the isolated cryoglobulin (*vide supra*) contained no traces of adsorbed impurities and migrated at the same speed as in the whole serum (Figure I).

TABLE III

Coagulation Studies.

Test	Before Extraction of Cryoglobulin	After Extraction of Cryoglobulin
Estimation of prothrombin time (Quick)	22 seconds (normal)	22 seconds
Thromboplastin Generation Test[1]	Result normal	Result normal
For fibrinolysin[2]	Absent	Absent

[1] The thromboplastin generation test was performed by the method of Biggs and Douglas (1953), a normal control's platelets being used.
[2] The Macfarlane dilution test (Firkin, Reed and Blackburn, 1957) was used to detect fibrinolysis.

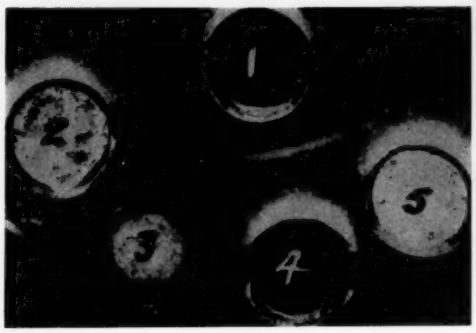

FIGURE III

Cup 1 contains anticryoglobulin serum, cup 2 eluted beta globulin, cup 3 eluted cold agglutinin, cup 4 cryoglobulin and cup 5 gamma globulin. Only one line of precipitate has formed between cups 1 and 4. This shows that the eluted cold agglutinin did not immunologically resemble cryoglobulin

Immunological Studies

Figure II shows that a cold agglutinin present in the patient's serum after the extraction of the cryoglobulin was a gamma globulin. Cup

I contains human gamma globulin antiserum, cup 2 the eluted cold agglutinins, cup 3 extracted cryoglobulin, and cup 4 eluted gamma globulin. The band formed between cups 1 and 2 runs into and joins the bands between cups 1 and 3 and cups 1 and 4. This is the so-called reaction of identity (Oudin, 1952), and means that an antigen present in cups 2 and 3 is immunologically identical with one present in Cup 4,—

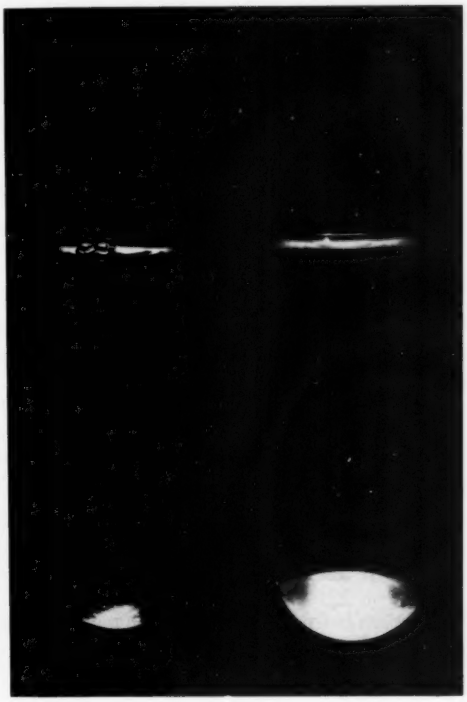

FIGURE IV

Right hand test tube contains patient's whole plasma diluted with saline and clotted with thrombin. Left hand tube contains patient's plasma from which the cryoglobulin has been extracted but otherwise treated similarly. Note the absence of fibrin clot in left hand tube containing the extracted plasma

that is, gamma globulin. The band between cups 1 and 3 was the last to develop and by far the weakest, indicating the low concentration gamma globulin present in the cryoglobulin extract.

The patient's cryoglobulin antiserum contained at least two antibodies. The antiserum gave one strong precipitate band within 48 hours when run against the patient's washed cryoglobulin (Figure III) but when the plate was kept for 10 days and the cup containing the cryoglobulin refilled, a faint band developed closer to the cup containing the cryoglobulin, and this band was shown to give a reaction of identity with a similar band formed by eluted gamma globulin. It was thought that this low titre of antibody to gamma globulin was due to adsorption of a small quantity of gamma globulin to the cryoglobulin which was not removed by washing with saline (Figure II).

The curve of the band of precipitate due to the cryoglobulin-anti-cryoglobulin complex was concave towards the cryoglobulin anti-sera (Figure III), and indicates that the molecular weight of the isolated cryoglobulin is less than that of its antibody. This means that the cryoglobulin must have a molecular weight within the normal globulin range unless the antibody was a macroglobulin (Korngold and van Leeuwin, 1957). No reaction was obtained when the cryoglobulin antiserum was tested against albumin, alpha-2 and beta globulins, Bence-Jones proteose, macroglobulin, myeloma proteins or cryoglobulins from two other sera.

Two antibodies were present, therefore, in the cryoglobulin antiserum; one (by far the stronger) was specific for the cryoglobulin, and the other (in very low titre) for gamma globulin.

Coagulation Studies

The coagulation studies are set out in Table III. The only abnormality discovered was the removal of a quantity of fibrinogen from the plasma after extraction of the cryoglobulin (Figure IV). A similar finding in a patient with essential cryoglobulinæmia has recently been reported (Firkin, 1958).

DISCUSSION

The long story that our patient gave of bouts of abdominal pain, recurrent hæmolytic episodes following exposure to cold, and Raynaud's phenomenon was typical of the syndrome of hæmolytic anæmia due to cold agglutinins. The severity of the abdominal pain in this patient is emphasized by its determining his first two admissions to hospital. There were only two findings which were at all anomalous for the diagnosis of acquired hæmolytic anæmia of the cold agglutinin type—first, the history of a bruising tendency following minor trauma, and secondly, the presence of splenomegaly. Pisciotta (1955) has emphasized that the latter finding in this condition should always lead to an intensive search for some other accompanying abnormality. The association of cryoglobulinæmia and cold agglutinins in this patient would probably have been overlooked but for the large amount of cryoglobulin present (Figure I), since we were unaware of the possibility of this association at the time. Indeed, only three such

cases had been reported in 1955 (Conn, 1955), and only recently have Christenson *et alii* (1957) recorded this combination in three out of 10 cases of cold hæmoglobinuria. It is possible that this combination may be more common than has previously been supposed, since it is only recently that the varying protein patterns in acquired hæmolytic anæmias have gained prominence (Christenson and Dacie, 1957). A routine search for cryoglobulins and other protein abnormalities is therefore mandatory in acquired hæmolytic anæmia, especially as the symptomatology of cryoglobulinæmia may be indistinguishable from that of cold-agglutinin hæmolytic anæmia.

The electrophoretic pattern of the patient's serum (Figure I) and the presence of the cryoglobulin immediately suggested the diagnosis of multiple myeloma or macroglobulinæmia. The bone-marrow smears examined nine months apart did not support either diagnosis. There were no bone changes to be seen on X-ray examinations of the skull, vertebræ and long bones, and no Bence-Jones protein could be found in the patient's urine even on immunological examination. These findings make the diagnosis of multiple myeloma improbable. The negative result of the Sia test is not against the diagnosis of macroglobulinæmia, especially when the abnormal protein migrates electrophoretically as a beta globulin (Laurell, Laurell and Waldenstrom, 1957). It is impossible in this instance to deny that the cryoglobulin was a macroglobulin without ultracentrifuge studies, which were unavailable; but the curvature of the line of precipitate obtained when the isolated cryoglobulin was run against its antiserum (Figure III) indicates that the cryoglobulin could be a macroglobulin only if its antiserum was also one (Korngold and van Leeuwin, 1957). It is also of interest that there was no cross-reaction between an isolated macroglobulin, the patient's cryoglobulin and the antiserum to the macroglobulin.

Protein abnormalities are frequently associated either numerically or morphologically with changes in the plasma cells; but examination of the bone-marrow in our patient revealed only a few plasma cells normal in structure and containing no inclusion bodies or vacuoles, which have been reported in some cases of cryoglobulinæmia (Firkin, 1958). The final diagnosis in this case was acquired hæmolytic anæmia of the cold-agglutinin type and essential cryoglobulinæmia.

Splenectomy was not undertaken, since the literature and personal experience have shown it to be of no specific value in cold hæmoglobinuria. The only indication for this operation would have been the presence of hypersplenism; but our patient was usually able to maintain his hæmoglobin level in spite of constant evidence of hæmolysis, and on his third admission to hospital responded to conservative measures of bed rest and warmth. Cortisone was given an extended trial of eight months, and did not reduce the level of cryoglobulin or alter the clinical course. The patient was particularly grateful for the advice given to avoid exposure to cold, and has noticed a great improvement in his general health since he has taken these precautions. However, despite his careful efforts, his last admission to the hospital was to combat hæmolysis.

Agglutinins

Agglutinins were demonstrated which were active against the red and white cells, but not against platelets. However, the latter finding lacks significance, since we have had no experience in the demonstration of these agglutinins. The red-cell agglutinins were most active at 4°C. (Table II), whereas the white-cell agglutinins were most active at 37°C. (Whyte and Yee, 1956). There was a great difference in cold-agglutinin activity after the extraction of the cryoglobulin, and the extracted cryoglobulin was found to contain a very high titre of cold agglutinin. In the case of the leucagglutinin, however, activity appeared to be relatively unchanged in the extracted serum, although the extracted cryoglobulin contained a higher leucagglutinin activity than was present in whole serum (Whyte and Yee). The red-cell cold agglutinin remaining after the removal of the cryoglobulin was shown to be a gamma globulin (Figure II). The great difference in cold-agglutinin titres before and after extraction of the cryoglobulin (Table II) may be ascribed to one of three mechanisms. The cryoglobulin itself may be a cold agglutinin, or the cryoglobulin may have acted as a potentiating factor in the phenomenon of agglutination, in the same way as polyvinyl pyrrolidone may potentiate agglutination in blood grouping. Finally, the cold agglutin may be adsorbed to the precipitated cryoglobulin in the same way as fibrinogen (Firkin, 1958). The greatly increased titre of cold agglutinin when the cryoglobulin extract was used favours the first and the last of the three theories, while the presence of gamma globulin (albeit of a very low titre) in the triply-washed isolated cryoglobulin makes the third theory the most likely.

The Cryoglobulin

The isolated cryoglobulin migrated electrophoretically between alpha and beta globulins (Figure I). It gave no reaction to lipid stains,

but stained heavily for carbohydrate in a similar manner to an abnormal myeloma protein (Osserman and Lawler, 1954).

Immunological studies (*vide supra*) showed that the cryoglobulin was unrelated to normal serum protein (Figure III), isolated beta and gamma myeloma proteins, Bence-Jones proteose, two other cryoglobulins and a macroglobulin. The cryoglobulin appeared to be a unique abnormal protein.

This patient's serum contained two abnormal proteins, the cryoglobulin and the cold agglutinin, and could be compared with myeloma serum containing a myeloma protein and a Bence-Jones proteose. Christenson, Dacie and Croucher (1957) have reported three patients with cold agglutinins and cryoglobulins, whose cold agglutinins and cryoglobulins were gamma-1 globulins. Finding that the agglutinin titre was reduced after extraction of the cryoglobulin, they state that "these results demonstrate clearly that the cold antibody can exist in the form of a cryoglobulin". Our studies indicate that this may not be true; the extracted cryoglobulin may merely adsorb the cold agglutinin. The fact that both cryoglobulin and cold agglutinin were gamma-1 globulins may not be significant, since all globulin electrophoretic components are heterogeneous in composition. In our case there can be no question of a direct structural relationship, although the properties of both the cryoglobulin and the cold agglutinin may have combined to produce the clinical manifestations.

It was thought that the patient's increased bruising tendency was related to the cryoglobulinæmia. Coagulation studies before and after extraction of the cryoglobulin revealed no abnormality, apart from a marked decrease in the amount of fibrinogen present after the extraction of the cryoglobulin (Table III and Figure IV). This phenomenon has been reported previously in another case of essential cryoglobulinæmia, and is thought to be due to the adsorption of the fibrinogen by the precipitated cryoglobulin. This may at least partly explain the bleeding diathesis often associated with cryoglobulinæmia (Firkin, 1958).

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A Review Written by Request

MACROGLOBULINS AND MACROGLOBULINÆMIA

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WALDENSTRÖM (1944, 1948) introduced the term "macroglobulinæmia" to describe the disease process characterized by a gross increase in circulating macroglobulins. Macroglobulins are serum globulins of high molecular weight, which are detectable by ultracentrifugal analysis of serum. Normally, macroglobulins comprise up to 4% of the total serum proteins and probably represent antibodies; the Wassermann antibody, the red-cell isoagglutinins, properdin and the rheumatoid factor are macroglobulins (Franklin *et alii*, 1957). A minor increase in the serum concentration of macroglobulins may occur in nephrosis, cirrhosis, lupus erythematosus and congenital syphilis (see Mackay, 1956), without *per se* causing any symptoms; this increase is of uncertain significance, but may reflect an augmented synthesis of antibody globulin. Strictly speaking, all the foregoing conditions could be regarded as examples of macroglobulinæmia, but by usage the term is limited to the clinical condition characterized by a gross and autonomous over-production of an anomalous macroglobulin component of the serum. Although macroglobulinæmia may occasionally occur as an isolated finding compatible with good health, it is usually associated with morbid effects.

In macroglobulinæmia, there are a generally recognized clinical picture, a well-defined biochemical disturbance (excessive macroglobulin synthesis), and a fairly constant histological appearance of infiltrations with cells of lymphocytic origin. Despite the possibility that more than one ætiological factor may be implicated, macroglobulinæmia is best regarded as a disease entity—there are recognized precedents for this in medicine such, for example, as sarcoidosis; in fact, the frequently debated question as to whether macroglobulinæmia should be regarded as a "syndrome" or a "disease entity" is merely a semantic issue. The increasing number of case reports of macroglobulinæmia can be attributed to the more general use of paper electrophoretic analysis of serum in clinical laboratories and to the increasing availability of ultracentrifugal analysis.

This review describes the more important clinical manifestations and relevant biochemical and pathological aspects of macroglobulinæmia, with an appraisal of our present concepts of this disease process. Details of 10 personally investigated cases are presented in Table I. A detailed documentation of macroglobulinæmia has been recently presented by Kappeler, Krebs and Riva (1958).

CLINICAL ASPECTS OF MACROGLOBULINÆMIA

Clinical Manifestations

Cases of macroglobulinæmia have been reported from Europe, North America, Israel and Australia, and the clinical picture as described by Waldenström (1948, 1952) has been confirmed. The disease occurs between the ages of 50 and 70 years, and seems to be more common in males than in females, although this sex incidence is not shown in the 27 cases collected by Quattrin *et alii* (1957). The major symptoms are weakness, lassitude, a bleeding diathesis, recurrent infections and disturbances of vision. The signs are those of anæmia, occasionally mild œdema, lymph-node enlargement, hepatosplenomegaly and eye-ground changes. These clinical features are determined by two factors: (*a*) systematized hyperplasia of cells of lymphocytic origin, causing marrow failure and enlargement of the liver, spleen and lymph nodes; (*b*) the physical presence of abnormal globulins in the blood, which may damage vascular endothelium and the surface of circulating cells, adsorb clotting factors, and

[1] Working with the aid of a grant from the National Health and Medical Research Council of Australia.

cause " sludging " of blood in retinal capillaries and cold-sensitivity phenomena. Clinical aspects of macroglobulinæmia of particular interest are discussed below.

Raynaud's Phenomenon and Cold Sensitivity.—Occasionally macroglobulins exhibit the properties of cryoproteins and precipitate on cooling. This may occur *in vivo*, resulting in acrocyanosis, Raynaud's phenomenon in the hands and peripheral gangrene on exposure of the extremities to cold. The literature relating to the occurrence of " cold allergy " (urticaria, œdema and asthma) in macroglobulinæmia has been well reviewed by Harders (1957).

Ocular Changes.—The ophthalmological findings in macroglobulinæmia are striking, and in some cases visual disturbance has been a major or even a presenting symptom (Ferriman and Anderson, 1956; Hanlon *et alii*, 1958). Retinal hæmorrhages, exudates and varicosities, with stasis and sludging of blood in retinal capillaries are seen on ophthalmological examination producing the so-called " fundus paraproteinæmicus " (Berneaud-Kötz and Nover, 1957). These effects are attributed to physical obstruction of blood flow by viscous macroglobulins in the retinal blood vessels. Harders (1957) has described similar changes in the conjunctival vessels.

Neuropsychiatric Symptoms.—Bizarre neurological disturbances (radiculitis, myelitis, encephalitis) sometimes associated with psychiatric changes have been reported in several cases of macroglobulinæmia, being referred to as the Bing-Neel syndrome (Bing and Neel, 1936; Mandema, 1954; Layani *et alii*, 1955; Mackay *et alii*, 1957). The manifestations can be attributed to infiltrations with lymphoid cells and protein exudates in the Virchow-Robin spaces in the central nervous system (Bichel *et alii*, 1950; Zollinger, 1958). A terminal delirium or coma (so-called " paraproteinæmic coma ") is ascribed to perivascular exudations in the brain (Wanner

TABLE I

Features of 10 Cases of Macroglobulinæmia[1, 2]

Patients Designation, Sex, Age (Years)	Duration of Symptoms, (years)[3]	Major Clinical Features	Bone Marrow Findings	Histological Findings[4]	Therapy[5]	References
SB f : 69	4 : d	Infections, pancytopenia, cerebral hæmorrhage	Plasma cells 5%	Non-specific	Splenectomy ±	Mackay *et alii* (1956)
RM m : 68	16 : d	Bleeding, hypertension, central nervous system signs, pancytopenia, congestive cardiac failure	Increased lymphocytes	Non-specific except for bone marrow lymphocytosis	Transfusions	Mackay *et alii* (1956)
CON m : 50	1¼ : d	Lympatic leukæmia, hæmorrhagic pleural effusion	Lymphocytes 80%	(S) Lymphosarcoma (no autopsy)	TEM + cortisone − splenectomy +	Mackay (1956) Mackay *et alii* (1957)
ENG f : 52	7	Bleeding, spider nævi, fractured spine, lachrymal enlargement, pancytopenia	Lymphocytes 81%	(S) Lymphosarcoma	P^{32} + +	Mackay *et alii* (1957)
BAR m : 56	6 : d	Neuropsychiatric disturbance, bleeding, pancytopenia, ? cerebral hæmorrhage	Lymphocytes 40%	(ln) Lymphosarcoma (no autopsy)	Nitrogen mustard +	Mackay *et alii* (1957)
JAM f : 52	4	Gross splenomegaly, anæmia, neutropenia; lasting remission following splenectomy	Lymphocytes 56%	(S) Lymphosarcoma	Splenectomy + +	Mackay *et alii* (1957)
LAN m : 51	4	Lachrymal and salivary gland enlargement; anæmia; 7% plasma cells in peripheral blood	Plasma cells 10%	(p) ? Lymphosarcoma, ? Mikulicz disease	P^{32} + +	Curtain (1959)
WO f : 73	15	Infections, anæmia	Lymphocytes 29% Plasma cells 2%	—	Prednisolone + +	Pitney *et alii* (1958)
GEN m : 74	1½ : d	Bleeding, anæmia, neutropenia, cerebral hæmorrhage	Lymphocytes 80%	Lymphosarcoma of lymph nodes and bone marrow	TEM +	
VIG m : 73	6 : d	Gross lymphadenopathy, anæmia	Non-specific	(ln) Lymphoma, indeterminate type (no autopsy)		

[1] Patients RM, WO and VIG were not examined personally.
[2] The concentration of macroglobulin in the 10 cases ranged from 1·7 to 7·5 grammes per 100 millilitres of serum; the electrophoretic mobility of the macroglobulin was that of a gamma globulin in nine cases and a beta globulin in one case; the sedimentation constant of the major macroglobulin component ranged from 12·9S to 17·5S; the Sia test result was positive in all cases except that of RM; the macroglobulin was cold precipitating in patients SB and VIG.
[3] d = patient has died of the disease.
[4] (S) = spleen, (ln) = lymph node, (p) = parotid gland.
[5] " ± " = of doubtful benefit; " + " = temporary benefit; " − " = no benefit; " + + " = good remission.

and Siebenmann, 1957), but may also result from cerebral bleeding.

Bone Changes.—The rarity of focal bone changes in macroglobulinæmia is an important point in the differentiation of the condition from multiple myeloma. At most there may be osteoporosis ; but bone pain, bony tenderness and focal X-ray translucencies seldom occur. This may be correlated with the diffuse nature of the cell infiltrations of macroglobulinæmia, in contrast to the focal nodular masses of plasmacytoma. Vertebral collapse due to osteoporosis has been described by Waldenström (1948), Layani *et alii* (1955) and others. In Hörner's case (1955), focal bone lesions were present, but the cell type was lymphocytic ; osteolytic foci in the femur were illustrated by Wanner and Siebenmann (1957) and have been described in a small number of other cases (see Kappeler *et alii*, 1958).

Renal Lesions.—Renal damage, attributed to the deposition of abnormal globulin in the glomeruli as paraamyloid, may occur in multiple myeloma. Jahnke and Scholtan (1954) and Schrade *et alii* (1958) have demonstrated that macroglobulins do not appear in significant concentration in the urine, although Bence Jones proteinuria is not infrequent. The rare occurrence of renal lesions in macroglobulinæmia was discussed by Schrade *et alii* (1958), although one of their four patients developed a nephrotic syndrome and renal insufficiency, with post-mortem evidence of paraamyloid in the kidney, liver and spleen. Thickening of the basement membrane of the glomeruli was described by Zollinger (1958).

Association of Macroglobulinæmia and Cancer.—Several authors, particularly Schaub (1953), have emphasized the coexistence of macroglobulinæmia with cancer ; the sites have included stomach, uterus, skin, larynx, biliary tract, lung and prostate. According to Kappeler *et alii* (1958), carcinoma was associated with macroglobulinæmia in 18 out of 146 reported cases. No definite statement can be made concerning the nature of this association, whether it is causal or coincidental. Wuhrmann (1952) has also referred to the coexistence of macroglobulinæmia and Sjøgren's syndrome.

Hæmatological Features.—In Waldenström's original description of macroglobulinæmia (1948), the characteristic mucosal bleeding was emphasized, but skin purpura was said not to occur, the syndrome of hyperglobulinæmia with skin purpura being regarded as a different entity. Purpura has not been stressed in subsequent reports, but may occur, particularly when the macroglobulin is also a cold-precipitating globulin or cryoglobulin. Bleeding, which may be massive, occurs from the nose, the mouth, the stomach (Quattrin *et alii*, 1956), the lower part of the gastro-intestinal tract (Voigt and Frick, 1956 ; Martin and Close, 1957), intracranially (Schaub, 1953 ; Mackay *et alii*, 1956 ; Hampton, 1958) and from sites of minor trauma.

Despite the fact that anæmia is a constant finding, the peripheral blood picture is often non-specific, although rouleaux formation may provide a hint to the diagnosis. The platelet and leucocyte counts may be low, and occasionally there is a mild lymphocytosis (see Mackay, 1956). Classically the bone marrow is rather densely infiltrated with lymphocytic cells, often with a scanty or absent cytoplasm. Despite the close resemblance of these cells to lymphocytes, many authors are doubtful as to their exact morphology and refer to them as " lymphocytoid " or " lymphoid ". Occasionally these cells bear some resemblance to small atypical plasma cells and raise the possibility of multiple myeloma. In some instances an increase in plasma cells in the marrow has accompanied the lymphocytic infiltration.

The hæmorrhagic tendency in macroglobulinæmia and other paraproteinæmias depends upon complex disturbances in the blood-clotting mechanism, for which any one of many possible causes may be responsible (see Kappeler *et alii*, 1958). Mechanisms implicated have included impaired platelet production due to marrow dysplasia, platelet damage by the macroglobulin molecules (Braunsteiner *et alii*, 1955), adsorption of various clotting factors, particularly fibrinogen, on the surface of paraproteins (Henstell and Kligerman, 1958 ; Firkin, 1958), inhibition of fibrin formation from fibrinogen (Lüscher and Labhardt, 1949 ; Jim and Steinkamp, 1956), an effect on prothrombin conversion factors (Long *et alii*, 1955), decreased A C globulin and prothrombin formation (Mandema, 1954 ; Jim and Steinkamp, 1956), damage to vascular endothelium by macroglobulins (Jahnke *et alii*, 1956), and mast cell hyperplasia with release of heparin (Tischendorf and Hartman, 1950).

Serum Protein Analysis.—Simple clinical laboratory determination shows a highly elevated serum protein level, the increase being in the globulin fraction, which may be over five or even ten grammes per 100 millilitres.

The Sia " euglobulin " test is the formation of a dense white precipitate when a drop of serum falls through a tube of distilled water. This simple test sometimes produces a negative result in macroglobulinæmia, and weak reactions may be obtained in other hyperglobulinæmias—

in fact, the reaction was originally described as a test for kala-azar; however, a positive response to the Sia test is suggestive of macroglobulinæmia. Other non-specific protein tests, such as the formol-gel reaction, also give positive results.

Filter-paper electrophoresis provides further diagnostic information. The globulin increase is seen to be due to a single homogeneous component, which stains as a dense discrete band in the gamma, or less commonly in the beta globulin area of the paper strip. Apart from multiple myeloma, very few conditions produce a discrete band; cryoglobulins may do so, and such bands on rare occasions have been detected in miscellaneous other conditions (Owen *et alii*, 1959).

Differential Diagnosis

The diagnosis of macroglobulinæmia should be considered in relation to elderly subjects regarded as suffering from atypical or obscure reticulosis, lymphoma or lymphatic leukæmia, particularly if mucosal bleeding is a prominent symptom. Laboratory findings of refractory anæmia, pancytopenia, bone-marrow lymphocytosis, increased serum globulin content, a greatly increased erythrocyte sedimentation rate and a positive response to the Sia test should suggest filter-paper electrophoresis of serum as the next step. A dense band resembling a myeloma component in the beta-gamma area is further evidence. Absolute confirmation requires ultracentrifugal analysis of serum.

If filter-paper electrophoresis has been performed, multiple myeloma is practically the only differential diagnosis to consider, and is distinguishable by the usual localization of myeloma to focal bone lesions, which are of a predominantly plasma-cell character, whereas in macroglobulinæmia extramedullary involvement is more common and a lymphocytic cell type predominates. In some cases there may be intermediate morphological cell forms diffusely infiltrating the bone marrow and extramedullary tissues; diagnosis in these cases may depend on the ultracentrifuge.

Confusion exists regarding the syndrome of purpura associated with a high globulin level, which has been described by Waldenström (1952) as "purpura hyperglobulinemica": this rather doubtful entity should be distinguished from macroglobulinæmia, for although the serum gamma globulin level is elevated, no discrete components are demonstrable by electrophoresis, and no macroglobulins by the ultracentrifuge.

Case reports occasionally appear in the literature with the diagnosis of "lymphoma", including lymphosarcoma and lymphatic leukæmia, associated with anomalous globulin components (Rundles *et alii*, 1954; Berndt, 1957; and others cited by Mackay, 1956); such cases could well be regarded as examples of macroglobulinæmia. Azar *et alii* (1957) and Ossermann (1957) described 13 cases of malignant lymphoma and lymphatic leukæmia associated with "myeloma type serum proteins". These authors did not cite ultracentrifugal data, but it seems likely that at least some of their patients were suffering from macroglobulinæmia.

Course and Prognosis

The course of macroglobulinæmia is very variable, although the disease is generally regarded as having an eventually fatal outcome (Schulten and Kanzow, 1956). In some cases, particularly those associated with a frankly neoplastic condition, the duration of life from the time of diagnosis is short, whereas in other instances, described as "asymptomatic macroglobulinæmia" by Kappeler *et alii* (1958), the condition appears to have been present for over ten years, occasionally without impairment of health.

The downward course is determined by neoplastic wasting and cachexia, intercurrent infections and sepsis, bleeding, and, rarely, cardiac insufficiency. Repeated infections, particularly pneumonia, are determined by neutropenia and a poor immune response due to a failure to manufacture normal antibody globulin. Bleeding usually means a poor prognosis; not infrequently death has resulted from a cerebral hæmorrhage. Occasionally death occurs from causes unrelated to macroglobulinæmia.

Treatment

Therapy, apart from supportive blood transfusions, is usually of palliative value only. Therapeutic procedures used have included the giving of ACTH or cortisone, splenectomy, deep X-ray therapy, and the administration of radio-active phosphorus (P^{32}) and the various cytotoxic drugs (urethane, nitrogen mustard, diamidine, triethylene melamine), with success ranging from nil to moderate. The outlook is, of course, best for those patients requiring no treatment.

Remissions of varying duration have been induced with corticosteroids, particularly when an autoimmune component is present (*vide infra* and Pitney *et alii*, 1959). Volpé *et alii* (1956) and Wirtschafter *et alii* (1956), in cases of cyroglobulinæmia, ascribed improvement to steroid therapy with a fall in cryoglobulin

concentration. Oxbrow (1957) observed an improvement in the retinal changes with prednisone therapy. Three of the patients seen at the Royal Melbourne Hospital derived temporary benefit from either nitrogen mustard or P^{32} therapy; lymphosarcoma had been suspected in all three. Combined therapeutic régimes have occasionally achieved success; improvement followed the use of cortisone with nitrogen mustard in cases reported by Ferriman and Anderson (1956) and Glencuhr *et alii* (1958). When enlargement of the spleen is associated with anæmia, the question of splenectomy will arise. In the case reported by Long *et alii* (1955), splenectomy restored the patient to health, whereas Jim and Steinkamp (1956) found splenectomy ineffective. Removal of an enormous spleen from one of our patients (JAM) induced a lasting remission and possibly a cure, since the macroglobulin is no longer demonstrable in the serum.

Physicochemical Studies on Macroglobulins

Physical Analyses

Macroglobulins, as well as myeloma proteins, impart a characteristic increase in viscosity to the serum, particularly on cooling (Jahnke *et alii*, 1958); according to Waldenström (1952), this may have diagnostic value. Macroglobulins may be precipitated when the serum is diluted with distilled water ("euglobulin" or Sia reaction) and also when the serum is cooled to below 20° C., which shows the macroglobulin to behave as a cryoglobulin. The precipitate is usually gelatinous or syrupy, in keeping with the mucoprotein nature of macroglobulins. Kratochvil and Deutsch (1956) have obtained a macroglobulin in crystalline form. Examination of a partially purified macroglobulin by electron microscopy revealed spherical particles measuring 120 to 180 Å (Gard *et alii*, 1952).

On moving-boundary electrophoresis, abnormal serum macroglobulins are characteristically homogeneous and migrate as a narrow, sharp, single peak or "spike" (contrast ultracentrifugal analysis) with a mobility varying from that of gamma to beta globulins. Very rarely macroglobulinæmia may be reflected in the electrophoretic pattern as a broad "hump" (Wilde and Hitzelberger, 1954), or as multiple components (Mackay *et alii*, 1956; Zlotnick, 1958). On zone electrophoresis in starch gel, macroglobulins fail to migrate through the medium; this property has been ascribed to the unusual configuration of these molecules, and may be of diagnostic value (Silberman, 1957). The sedimentation rate of components in the ultracentrifuge is expressed mathematically by the "sedimentation constant", which is defined in Svedberg or S units; this gives only a rough indication of molecular weight, since the S value is a function of molecular size, molecular shape and protein concentration in the ultracentrifuge cell. Although the macroglobulins in Waldenström's original cases sedimented at 17S, which was assumed to represent a molecular weight of about 1,000,000, numerous subsequent authors have accepted as "macroglobulins" 12S to 17S components, and even globulins sedimenting between 7S and 12S (Deutsch *et alii*, 1956). The concentration dependence of macroglobulins in the ultracentrifuge (Kratochvil and Deutsch, 1956) makes comparisons of the sedimentation behaviour of variously reported macroglobulins difficult, and invalidates the stipulation of Martin and Close (1957) that "macroglobulinæmia" should be diagnosed only when the abnormal component has a sedimentation constant of 17S or greater. Rarely, the major abnormal serum component has an S value between that of gamma globulin (7S) and 12S (Motulsky *et alii*, 1957; "atypical macroglobulinæmia" of Jahnke and Scholtan, 1955); in such cases the diagnosis must (if possible) be established on grounds other than ultracentrifugal analysis.

The electrophoretic homogeneity of macroglobulins is not borne out in the ultracentrifuge, since multiple components are usually present (Petermann and Braunsteiner, 1956). However, the schlieren pattern does show a sharp major peak indicative of the atypical nature of these globulins. Physical analysis in one of our earlier cases (Mackay *et alii*, 1956) led us to suggest that some macroglobulins might represent molecular aggregates of gamma globulins rather than being separate protein species. The addition of sulphydryl compounds results in disaggregation of macroglobulins to monomer units with an S value of 6·5 (Deutsch and Morton, 1957; Glenchur *et alii*, 1958) with a marked decrease in serum viscosity (Isliker, 1958), suggesting that some macroglobulins may be polymers of the normal gamma globulins.

Biochemical Structure of Macroglobulins

Chemical analyses of macroglobulins have been concerned with total amino acid composition, amino end-group analysis and carbohydrate content. Although the amino-acid composition of paraproteins varies from patient to patient, the general pattern shows a close similarity to that of normal gamma globulin; a low proline content was reported by Mandema *et alii* (1955), a low lysine content

by Pernis *et alii* (1954) and hydroxyproline, normally absent from serum proteins, was detected in one cryomacroglobulin (Mandema *et alii*, 1956). Other relevant literature has been cited by Mackay *et alii* (1956) and Putnam (1957).

Amino end-group analysis has been applied by Putnam (1957) mainly to myeloma globulins (6 S to 8 S); four distinct groupings and a fifth heterogeneous group were defined. The myeloma globulins included a macroglobulin and showed definite differences in end-group amino acids from normal gamma globulin; their chemical individuality was considered by Putnam (1957) to support immunological data that these globulins were truly aberrant proteins.

The high polysaccharide content of certain myeloma globulins and macroglobulins is most simply demonstrated by periodic acid-Schiff (P.A.S.) staining of paper electrophoretic strips (Di Guglielmo and Antonini, 1955; Laurell *et alii*, 1957; Isliker, 1958), and is also reflected by highly elevated serum concentrations of hexosamine (Mackay *et alii*, 1957; Laurell *et alii*, 1957). The hexosamine levels in four cases of macroglobulinæmia studied in our Unit by Weiden (1958) were 260, 284, 286 and 173 milligrammes per 100 millilitres, our normal range being 80 to 124 milligrammes per 100 millilitres. Laurell *et alii* (1957) found that serum concentrations of hexose, hexosamine, fucose, sialic acid and total polysaccharide were usually greatly in excess of normal values, and the abnormal macroglobulin component made the outstanding contribution to these levels. Macroglobulins contain up to 10% carbohydrate with a sialic acid content eight times that of normal gamma globulin, and according to Müller-Eberhard *et alii* (1956) the disproportionately high carbohydrate content of macroglobulins is evidence against their being simple molecular aggregates of gamma globulin. Although macroglobulins regularly showed a high polysaccharide content, only faster-moving myeloma globulins did so (Laurell *et alii*, 1957); this was considered to be of possible diagnostic value.

The presence of mucoprotein containing "sialic acid" (for nomenclature see Blix, Gottschalk and Klenk, 1957) in macroglobulinæmia sera was indicated by some observations of Dr. E. L. French (1956) of this Institute, using a biological method. Certain mucoproteins, including mucoprotein(s) present in normal serum inhibit hæmagglutination by heat-inactivated influenza viruses ("indicator viruses", Stone, 1949). This inhibitory effect of the mucoproteins is lost when they are pretreated with an enzyme present in the culture filtrate of *Vibrio choleræ* (receptor destroying enzyme, RDE) or with living influenza virus. RDE has been recently identified as neuraminidase, the specific substrate of which is a disaccharide present in mucoproteins containing sialic acid (Gottschalk, 1956, 1958). Table II shows that macroglobulinæmic sera inhibit hæmagglutination by "indicator viruses" in extremely high titre, and inhibition is completely abolished by treatment of the sera with RDE (neuraminidase); this evidence for a high mucoprotein content correlates well with the chemically determined levels of hexosamine in these sera.

TABLE II

Inhibition of Hæmagglutinin Activity of Heated Influenza "Indicator" Viruses (Lee and WSE) Before and After Treatment with RDE (Neuraminidase)

Serum	Inhibition Titre			Serum Hexosamine (milligramme per 100 millilitre)
	Serum before RDE		Serum after RDE	
	Virus Lee	Virus WSE	Virus Lee and WSE	
BAR	400000	240000	<10	284
ENG	240000	240000	<10	286
JAM	160000	140000	<10	173
Normal	1400	400	<10	100

Immunochemistry of Macroglobulins

There is good evidence that individual macroglobulins are immunologically "distinct" both from normal serum components and from each other. Specific immune antisera were prepared in rabbits against isolated macroglobulins from 16 patients by Habich (1953); besides demonstrating group specificity for macroglobulins, Habich found that individual macroglobulins also reacted specifically to their own antiserum. The immunological individuality of macroglobulins has been corroborated by Kanzow *et alii* (1955), Westendorp Boerma and Mandema (1957), Korngold and Van Leeuwen (1957), Sehon and co-workers (Rose *et alii*, 1956; Sehon *et alii*, 1957) and Scheidegger *et alii* (1958). Analogous findings for myeloma proteins were reported by Slater *et alii* (1955).

On the other hand, Deutsch *et alii* (1956) and Morton and Deutsch (1958) showed that macroglobulins did not contain any determinants absent from normal serum, and therefore argued that macroglobulinæmia represented a quantitative elevation of a specific component normally occurring at a low level in serum rather than the occurrence of a new molecular species. The immunological studies of Cleve and Schwick (1957) also suggested that prototypes for macro-

globulins of macroglobulinæmia existed in normal serum. Martin and Close (1957) appear to take a similar view, and would reject terms such as " paraproteins ".

Thus, expert protagonists are found for both sides of the question; however, the recent weight of evidence favours Putnam's view (1957) that myeloma globulins, cryoglobulins and macroglobulins are indeed " truly abnormal proteins, the products of an abnormal cell with a perverted mechanism of protein synthesis "; it would thus seem that " paraprotein " is as good a term as any to describe such globulins. The problem " is not academic " according to Putnam, who leans to my previously expressed somatic mutation hypothesis (Mackay, 1956) as the explanation of paraprotein synthesis. Waldenström (1958) has also expressed support for this viewpoint.

Antibody Properties of Macroglobulins

In some cases abnormal macroglobulins may behave as antibodies. It is known (*vide supra*) that certain well-defined antibodies (iso-agglutinins, Wassermann antibodies and complete Rh agglutinins) are associated with the normally occurring high molecular weight gamma globulin. The rheumatoid factor responsible for the sensitized sheep-cell agglutination or Rose-Waaler reaction is also a macroglobulin complex (Svartz, 1957, Franklin *et alii*, 1957), although its presence is never associated with the features of macroglobulinæmia.

Some observations relevant to antibody properties of certain macroglobulins have been carried out in this Institute. Two out of 14 macroglobulinæmia sera have exhibited a very high titre of complement-fixing activity with human tissue antigens in the autoimmune complement fixation (A.I.C.F.) reaction (Gajdusek, 1958; Mackay and Larkin, 1958). Studies on the immunological reactivity of the sera of the two patients (JAM and WO) are tabulated below (Table III).

The serum JAM reacted to extremely high titre with various organ antigens, except for extracts of the patient's own tissues; the implications of the latter finding have been discussed by Mackay and Larkin (1959). The macroglobulin was isolated by fourfold dilution in distilled water, with successive resolution in isotonic saline; the final solution, containing approximately 200 milligrammes of protein per 100 millilitres, was over 80% homogeneous, albumin being the main contaminant. Macroglobulin was also isolated by elution of the macroglobulin band following paper strip electrophoresis. The A.I.C.F. activity of the serum was shown to reside exclusively in the isolated component; secondly, the macroglobulin did not have normal antibody properties, for it was not associated with the anti-influenza, antistaphylococcal and antiherpes antibody present in the whole serum.

After removal from JAM of a spleen weighing 2·7 kilogrammes and showing the histological picture of lymphosarcoma, the response to the Sia test became negative, the macroglobulin was no longer electrophoretically demonstrable and was barely detectable on ultracentrifugal analysis, and the A.I.C.F. titre fell from 32000 to <4.

The second patient, WO, whose case was reported in detail by Pitney *et alii* (1958), had a serum A.I.C.F. titre of 1024. Treatment with prednisolone, which corrected a refractory anæmia, resulted in a sharp fall in concentration of the paraprotein, as judged by its disappearance from the paper electrophoretogram and a marked reduction in size of the macroglobulin peak in the ultracentrifuge pattern (see Pitney *et alii*, 1958). Since there was a concomitant fall in the A.I.C.F. titre from 1024 to <4, it could be assumed that the A.I.C.F. activity in this serum, as in the case of

TABLE III

Immunological Reactivity of Macroglobulinæmia Sera[1]

Antigens	Antibody Titre of Sera				
	JAM Serum	JAM Isolated Macroglobulin	JAM serum after Splenectomy	WO	
				Before Prednisolone	After Prednisolone
Human tissues (isologous)	8000 −32000	8000	<4	1028	<4
JAM tissues (autologous)	<4	—	—	—	—
Staphylococcal toxin	128	<2	—	—	—
Influenza type A	20	<5	—	—	—
Influenza type B	10	<5	—	—	—
Herpes febrilis egg-adapted	present	absent	—	—	—

[1] "—", not tested.

JAM, resided in the macroglobulin component. The striking fall in antibody titre following prednisolone therapy is in keeping with the suppressive effects of corticosteroids on antibody formation (Scheiffarth *et alii*, 1956; Colucci, 1957; Taliaferro, 1957).

One of 12 myeloma sera exhibited a high A.I.C.F. titre (1024); studies on the relationship of the antibody activity to the myeloma component of this serum were inconclusive.

Cryoglobulins and macroglobulins have been associated with the syndrome of hæmolytic anæmia of the cold-agglutinin type and Raynaud's phenomenon; in certain of these cases steroid therapy was of considerable benefit (Craig *et alii*, 1952; Conn, 1955; Christenson and Dacie, 1957; Fudenberg and Kunkel, 1957; Gaddy and Powell, 1958). In one instance (Forster and Moeschlin, 1954), the antibody was of the warm type.

In the case reported by Firkin *et alii* (1959) in this journal, cryoglobulins, a cold hæmagglutinin and a white-cell agglutinin were demonstrable in the patient's serum; the cryoglobulin itself was probably responsible for the cold-agglutinin activity of the serum, for when isolated by cold precipitation, the cryoglobulin contained a very high titre of cold agglutinin. Residual hæmagglutinin activity remaining in the serum after extraction of the cryoglobulin (at 4°C.) would not necessarily imply the existence of a second abnormal serum globulin, since cryoglobulins are not entirely precipitated at 4°C. (Mackay *et alii*, 1956). Cold agglutinins have been identified as an 18 S macroglobulin (Gordon, 1953; Weber, 1956), and both Christenson *et alii* (1957) and Fudenberg and Kunkel (1957) showed that the antibody causing cold hæmagglutination existed in the form of a circulating macroglobulin.

Wirtschafter *et alii* (1956) discussed "the similarities between cryoproteins and immune bodies" in connexion with a patient with "cold allergy" associated with cryoglobulinæmia; antibody activity of the isolated cryoprotein was, however, not demonstrated. As in the patient WO (*vide supra*), the paraprotein in Wirtschafter's case was greatly reduced after treatment with cortisone. Bohrod (1957) described a transient plasmacytosis and cryoglobulinæmia in a patient with a cancer of the breast, and postulated that this might have represented an immunological response, possibly to the cancer itself. Volpé *et alii* (1956) found a remarkably high anticomplementary titre of the serum in a case of essential cryoglobulinæmia, and showed this activity to be resident entirely in the cryoglobulin fraction.

THE PATHOLOGY OF MACROGLOBULINÆMIA

Histological Characteristics

The underlying disorder in macroglobulinæmia is a systematized proliferation of cell components of the lymphoid system, resulting in enlargement of the spleen and lymph nodes, and in bone-marrow lymphocytosis and accompanying marrow failure. Dense infiltrations of lymphoid cells may be present in the periportal zones of the liver (Wuketich and Siegmund, 1958; Long *et alii*, 1955), in the kidney (Zollinger, 1958), and throughout other viscera (Braunsteiner *et alii*, 1954). However, the histological appearances of the cellular proliferations may be quite variable, and thus no unanimity exists as to the exact morphological basis of the disease; histopathological diagnoses cited in published cases have been listed elsewhere by Mackay *et alii* (1957) and Kappeler *et alii* (1958).

The proliferating cells frequently resemble small lymphocytes, but may resemble atypical plasma cells or intermediate or transitional cell types (Motulsky *et alii*, 1957). Occasionally both lymphocytic and plasma cells may be recognized; the cell type may seemingly change during the course of the disease (Schaub, 1952). The infiltrations are rarely in clumps or aggregates in the bone-marrow, and the absence of localized bony rarefactions helps differentiate macroglobulinæmia from classical multiple myeloma. The proliferation is often clearly autonomous, the histological changes being those of lymphoma, particularly lymphosarcoma, or diffuse plasmacytoma; when the process has been less obviously malignant, it has been referred to in the literature as a "leucosis", "lymphadenosis" or "plasmacytosis". The changes have been accepted as essentially neoplastic by several authors including Schaub (1952), Lennert (1955) Schulten and Kanzow (1956), Braunsteiner *et alii* (1956), Lelbach (1957) and Dutcher and Fahey (1958); Kappeler *et alii* (1958) express a current European viewpoint in defining Waldenström's macroglobulinæmia as a "*lymphoid-plasmacelluläre Reticulomatose mit neoplastischem Charakter*". Macroglobulinæmia has been occasionally reported in the absence of definite histological abnormalities. The significance of the increase in mast cells in the bone-marrow and elsewhere in macroglobulinæmia (Tischendorf and Hartman 1950; Hampton 1958), is uncertain.

Sites of Macroglobulin Synthesis

Current opinion on the cellular morphology of immune globulin synthesis (Burnet, 1959) emphasizes the cardinal role of the plasma cell

in antibody production, particularly in the acute phase of the immune response. Strong evidence for this has been provided by the application of fluorescein-labelled rabbit anti-human globulin to sections of lymph node and bone-marrow (Coons *et alii*, 1955; Ortega and Mellors, 1957). It is probable, but less certain, that the small lymphocyte is also concerned in immune globulin production. The normally occurring macroglobulins are closely identifiable with antibody globulins and are presumably synthesized by similar cell types. There is considerable presumptive evidence (Wollensak and Seybold, 1956; Putnam, 1957) that the malignant plasma cell is actually the source of myeloma protein, and confirmatory evidence based on the fluorescent antibody technique has been obtained by Wollensak and Seybold (1956) and Vasquez (1958).

Less certainty exists concerning the cell type responsible for the synthesis of abnormal macroglobulins. In a single case, Abrams *et alii* (1949) extracted a macroglobulin from lymphosarcoma tissue and identified it with a similar protein in the circulating blood. From the histological viewpoint, the proliferating cells in macroglobulinæmia frequently exhibit the presence of pyronine-staining or P.A.S.-staining material in the cell cytoplasm (Schamaun, 1954; Jim and Steinkamp, 1956; Martin and Close, 1957; Azar *et alii*, 1957; Wuketich and Siegmund, 1958; "grape cells" of Zlotnik, 1958) or within the cell nucleus (Dutcher and Fahey, 1958); these staining reactions are predominantly features of the plasma cell, and are histochemical reflections of gamma globulin synthesis. Pyroninophilia is not a specific property of plasma cells, however, being also exhibited by lymphoid cells and reticulum cells, as in the cases of lymphoma reported by Azar *et alii* (1957). Rappaport and Johnston (1955) have described P.A.S.-staining material resembling Russell bodies in malignant lymphoma cells in cases of lymphoma associated with autoimmune hæmolytic anæmia; they considered that this material was being formed by, rather than taken up by, the neoplastic lymphocytes, and might have been a precursor of circulating autoantibody. In the electron microscope studies of Braunsteiner *et alii* (1957), protein synthesis was correlated with a cytoplasmic structure, the ergastoplasm, which was specifically present in plasma cells; macroglobulinæmia was the only condition in which ergastoplasm was present in cells of lymphoid character. On present evidence, it would appear that abnormal macroglobulins as well as other gamma globulins may be synthesized by cells with the morphology of either lymphocytes or plasma cells.

Curtain's studies, reported in this issue, concerning sites of macroglobulin synthesis using the fluorescent antibody technique are of considerable interest. In his patient A, he stained bone-marrow smears with a fluorescein-labelled rabbit antimacroglobulin serum; his results suggested that plasma cells in the bone marrow, which on conventional staining were increased in number and showed evidence of synthetic activity, were responsible for macroglobulin synthesis. However, the opportunity was not provided for studying the parotid lesion by the same technique in order to determine whether the lymphocytes invading the parotid were also synthesizing protein.

MACROGLOBULINÆMIA—A CLONAL PHENOMENON

Macroglobulinæmia may be best classified with that group of conditions which are consequent upon some pathological modification of the cell system subserving antibody production and the immune response. This group includes both frankly autonomous and neoplastic proliferations (lymphomas, myeloma), and also doubtfully neoplastic widespread proliferations best designated by terms such as "reticulosis" or "leucosis". The nature of this pathological modification is presumed to be a somatic mutation with preferential proliferation of descendents of the mutant cell throughout the reticulo-endothelial system. The rapid rate of cell turnover in the lymphoid tissues would naturally favour the development of such mutations. The retention of functional activity by the cell types involved would lead to the synthesis of a variety of pathological globulins, manifested by macroglobulinæmia, cryoglobulinæmia, primary amyloidosis and auto-immune globulin production, whereas abrogation of function might result in acquired hypogammaglobulinæmia.

The "clonal" concept of antibody production recently elaborated by Burnet (1957, 1959) is highly relevant to the elucidation of macroglobulinæmia. In his clonal selection hypothesis, Burnet suggests that antigenic determinants and their antibody globulins are represented in the lymphoid tissues by a corresponding clone of lymphocytic cells; under conditions of active antibody production to a particular antigenic determinant, these corresponding cells proliferate, forming a clone of genetically identical cells capable of synthesizing that one single species of antibody molecule. Nossal's work (1958) on antibody production by single cells is in keeping with the clonal concept.

The synthesis of abnormal macroglobulins may be similarly regarded as a clonal phenomenon. The physicochemical properties and antigenic "uniqueness" displayed by macroglobulins imply genetic individuality at a cellular level; in other words, each abnormal macroglobulin is synthesized by cells of an identical genotype constituting a single cell clone within the lymphocyte-plasma cell system. To refer again to Curtain's studies, two distinct paraproteins were identified in the serum of his patient B, a major myeloma protein component and a minor macroglobulin component. Fluorescein-labelled antibodies, prepared to each of these paraproteins, were used to stain bone-marrow smears; different plasma cells were apparently responsible for the synthesis of the two abnormal proteins and for normal gamma globulin. Curtain has not provided detailed evidence for the immunological specificity of the antisera which he prepared to the two paraproteins isolated from serum B, although he did demonstrate that pretreatment of marrow smears with high-titre antibody to myeloma protein suppressed fluorescence with the labelled anti-myeloma antibody, but not with labelled antimacroglobulin antibody, and vice versa. Fluorescein-labelled antibody studies are inherently difficult and must be interpreted with caution; Curtain's evidence does suggest, however, that when different paraproteins are present in serum, they may be synthesized by different groups (or clones) of cells.

When the mutant clone assumes frankly neoplastic properties, it may give rise to the histological picture of myeloma, malignant lymphoma or leukæmia; the occurrence of an associated paraproteinæmia in such instances has already been discussed. However, there may be instances, particularly in elderly subjects, when descendents of the mutant clone are not eliminated, yet the process does not progress to frank malignancy; macroglobulinæmia may then be demonstrable over a considerable period as the only observable abnormality. Although macroglobulin synthesis is proceeding in an apparently autonomous fashion, evidence for an underlying neoplasm or reticulosis is lacking. If the assumed mutation has in fact taken place, a further transformation towards malignancy would then be anticipated in such individuals.

Curtain has suggested that macroglobulinæmia may represent a "reactive process", or a type of immunological response, another manifestation of which may be the plasmacytosis of the bone marrow. Moreover, macroglobulins occasionally exhibit autoantibody-like properties. This response may represent an autoimmune reaction to unusual antigenic materials, such as components of specialized glands (e.g., parotid and lachrymal glands) and malignant tissues. The well-documented coexistence of macroglobulinæmia with neoplasms and with Sjøgren's disease may be relevant in this context. A review of the histological material from Curtain's Patient A, generously provided by Dr. Curtain and Dr. R. Motteram, did in fact suggest the possibility of a parotid involvement of the Mikulicz-Sjøgren type.

The chief objection to the "reactive" concept lies in the relative homogeneity of macroglobulins ("spike" components) on physicochemical analysis. It is more probable that if pathological globulins (or the cells producing them) are immunologically reactive with tissue components, this is essentially an accidental epiphenomenon, rather than representing a conditioned response to a particular antigenic determinant.

CONCLUSIONS

Macroglobulinæmia is a disease entity characterized by reticuloendothelial hyperplasia or neoplasia and a gross increase in concentration of abnormal serum globulin of high molecular weight, demonstrable by electrophoresis and ultracentrifuge analysis. Manifestations of this disease, which may occur singly or in combination, include a bleeding diathesis, pancytopenia and marrow failure, a lymphoma-like syndrome and hæmolytic anæmia of the cold agglutinin type.

Macroglobulins may exhibit the properties of antibodies, particularly autoantibodies. It is considered, however, that macroglobulin synthesis is essentially an autonomous rather than a reactive process, and the occasional presence of immunologically reactive sites on macroglobulin molecules must be regarded as an incidental phenomenon.

The underlying pathological process in macroglobulinæmia is a proliferation of those elements of the mesenchymal tissues responsible for the synthesis of immune globulins. Both lymphocytes and plasma cells may participate in this process, but the predominant cell type is of lymphocytic origin. The antigenic and physicochemical individuality of macroglobulins and other paraproteins has led to the supposition that they are products of clones of genetically identical cells within the antibody-producing system. Such a clone may arise through a mutation process, and ultimately assume frankly neoplastic properties, although occasionally

in antibody production, particularly in the acute phase of the immune response. Strong evidence for this has been provided by the application of fluorescein-labelled rabbit anti-human globulin to sections of lymph node and bone-marrow (Coons *et alii*, 1955; Ortega and Mellors, 1957). It is probable, but less certain, that the small lymphocyte is also concerned in immune globulin production. The normally occurring macroglobulins are closely identifiable with antibody globulins and are presumably synthesized by similar cell types. There is considerable presumptive evidence (Wollensak and Seybold, 1956; Putnam, 1957) that the malignant plasma cell is actually the source of myeloma protein, and confirmatory evidence based on the fluorescent antibody technique has been obtained by Wollensak and Seybold (1956) and Vasquez (1958).

Less certainty exists concerning the cell type responsible for the synthesis of abnormal macroglobulins. In a single case, Abrams *et alii* (1949) extracted a macroglobulin from lymphosarcoma tissue and identified it with a similar protein in the circulating blood. From the histological viewpoint, the proliferating cells in macroglobulinæmia frequently exhibit the presence of pyronine-staining or P.A.S.-staining material in the cell cytoplasm (Schamaun, 1954; Jim and Steinkamp, 1956; Martin and Close, 1957; Azar *et alii*, 1957; Wuketich and Siegmund, 1958; "grape cells" of Zlotnik, 1958) or within the cell nucleus (Dutcher and Fahey, 1958); these staining reactions are predominantly features of the plasma cell, and are histochemical reflections of gamma globulin synthesis. Pyroninophilia is not a specific property of plasma cells, however, being also exhibited by lymphoid cells and reticulum cells, as in the cases of lymphoma reported by Azar *et alii* (1957). Rappaport and Johnston (1955) have described P.A.S.-staining material resembling Russell bodies in malignant lymphoma cells in cases of lymphoma associated with autoimmune hæmolytic anæmia; they considered that this material was being formed by, rather than taken up by, the neoplastic lymphocytes, and might have been a precursor of circulating autoantibody. In the electron microscope studies of Braunsteiner *et alii* (1957), protein synthesis was correlated with a cytoplasmic structure, the ergastoplasm, which was specifically present in plasma cells; macroglobulinæmia was the only condition in which ergastoplasm was present in cells of lymphoid character. On present evidence, it would appear that abnormal macroglobulins as well as other gamma globulins may be synthesized by cells with the morphology of either lymphocytes or plasma cells.

Curtain's studies, reported in this issue, concerning sites of macroglobulin synthesis using the fluorescent antibody technique are of considerable interest. In his patient A, he stained bone-marrow smears with a fluorescein-labelled rabbit antimacroglobulin serum; his results suggested that plasma cells in the bone marrow, which on conventional staining were increased in number and showed evidence of synthetic activity, were responsible for macroglobulin synthesis. However, the opportunity was not provided for studying the parotid lesion by the same technique in order to determine whether the lymphocytes invading the parotid were also synthesizing protein.

MACROGLOBULINÆMIA—A CLONAL PHENOMENON

Macroglobulinæmia may be best classified with that group of conditions which are consequent upon some pathological modification of the cell system subserving antibody production and the immune response. This group includes both frankly autonomous and neoplastic proliferations (lymphomas, myeloma), and also doubtfully neoplastic widespread proliferations best designated by terms such as "reticulosis" or "leucosis". The nature of this pathological modification is presumed to be a somatic mutation with preferential proliferation of descendents of the mutant cell throughout the reticulo-endothelial system. The rapid rate of cell turnover in the lymphoid tissues would naturally favour the development of such mutations. The retention of functional activity by the cell types involved would lead to the synthesis of a variety of pathological globulins, manifested by macroglobulinæmia, cryoglobulinæmia, primary amyloidosis and auto-immune globulin production, whereas abrogation of function might result in acquired hypogammaglobulinæmia.

The "clonal" concept of antibody production recently elaborated by Burnet (1957, 1959) is highly relevant to the elucidation of macroglobulinæmia. In his clonal selection hypothesis, Burnet suggests that antigenic determinants and their antibody globulins are represented in the lymphoid tissues by a corresponding clone of lymphocytic cells; under conditions of active antibody production to a particular antigenic determinant, these corresponding cells proliferate, forming a clone of genetically identical cells capable of synthesizing that one single species of antibody molecule. Nossal's work (1958) on antibody production by single cells is in keeping with the clonal concept.

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macroglobulinæmia may continue to be the sole evidence of the existence of the aberrant clone.

A plea is made for an awareness of macroglobulinæmia on the part of our physicians, surgeons and pathologists, for it will be evident that recognition of additional cases and a closer understanding of this condition will provide clearer insight into problems concerned with antibody production, somatic mutation and cancer.

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